Beneficial effects of blackcurrant and apple polyphenols on glucose homeostasis

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Beneficial effects of blackcurrant and apple polyphenols on glucose homeostasis

A thesis submitted by

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A mis padres, hermanos y sobrinos

Si una montaña se interpone en tu camino, rodéala. Si no puedes rodearla, pasa por encima. Si no puedes pasar por encima, detente y pregúntate si vale la pena llegar al otro lado. En caso afirmativo, cava un túnel.

Publications and oral communications

Castro-Acosta, M. L., et al. (2016) Berries and anthocyanins: promising functional food ingredients with postprandial glycaemia-lowering effects. Proc Nutr Soc: (75), 342-355.

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Abstract

Epidemiological evidence and randomised controlled trials suggests that dietary patterns with high intakes of fruit and vegetables are associated with reduced risk of cardiovascular disease (CVD) and type 2 diabetes (T2D). Frequent elevated excursions in postprandial glucose concentrations are thought to increase risk of T2D, therefore dietary strategies to control chronic postprandial hyperglycaemia would strengthen efforts to reduce the risk for T2D. Fruit polyphenols are dietary constituents that might help to delay glucose absorption following a carbohydrate-containing meal or beverage and epidemiological studies have shown a negative relationship between ingestion of polyphenols and T2D. Polyphenol dietary intakes and urinary excretions were analysed in a free-living population following the UK dietary guidelines and compared with a population following the average UK diet. In the intervention group, increased consumption of fruit and vegetables ($\geq$ 5 portions/day) and wholegrain cereals led to an increased intake in polyphenols associated with health benefits, such as anthocyanins, isoflavones and lignans ($P<0.05$). Randomised clinical trials were conducted using highly purified anthocyanin-rich blackcurrant extract (BE) and polyphenol-rich apple extracts (AE) consumed immediately before a high-carbohydrate (starch and sucrose) test meal. BE (600 mg of blackcurrant anthocyanins), AE (1200 mg of apple polyphenols) and BE+AE (600 mg of blackcurrant anthocyanins + 600 mg of apple polyphenol) reduced postprandial glycaemia relative to control by 57, 63 and 73 %, respectively ($P<0.005$), but lower doses of an apple extract had no effect on postprandial responses to an oral glucose load. Effective doses of the fruit extracts are equivalent to 100 g of fresh blackcurrant and 600 and 300 g of raw apple. *In vitro* studies testing physiological concentrations of the blackcurrant and apple extracts used in the randomised clinical trials showed that the polyphenols contained in blackcurrant and apple extracts inhibited total and GLUT-mediated glucose uptake in Caco-2/TC7 cells (a well-known model of the small intestine) and inhibited the sodium-dependent glucose transporter SGLT1 expressed in oocytes. Our results suggest blackcurrant and apple polyphenols may reduce postprandial glycaemia *in vivo* at least partly by inhibiting glucose uptake in the small intestine. Altogether the findings presented show that advice to consume more fruits and vegetables can effectively increase intakes of specific polyphenols that have been associated with reduced risk of cardio-metabolic diseases, and provides evidence for mechanisms where fruit polyphenols might regulate glucose homeostasis.

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Abbreviations

24-HR	24-hour recalls
4-DFD	Four-day food diary
95% CI	95 % confidence interval
ACNs	Anthocyanins
AE	Apple extract
AMPK	AMP protein kinase
AOB	Area over baseline
AUC	Area under the curve
BE	Blackcurrant extract
BMI	Body mass index
BP	Blood pressure
CB	Cytochalasin B
cDNA	Complementary DNA
Cmax	Maximum concentration
CON	Control drink
CRESSIDA	The Cardiovascular risk REduction Study: Supported by an Integrated Dietary Approach
cRNA	Complementary RNA
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DMSO	Dimethyl sulfoxide
DRI	Dietary reference intake
DVP	Digital volume pulse
DVP-RI	Reflection index
DVP-SI	Stiffness index
eNOS	Endothelial nitric oxide synthase
EPIC	The European Prospective Investigation into Cancer and Nutrition
F&V	Fruit and vegetables
FFQ	Food frequency questionnaire
GC-MS	Gas chromatography mass spectrometry
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GLUT	Sodium-independent glucose transporter
GSK	GlaxoSmithKline
HDL	High density lipoprotein
HOMA-IR	Homeostatic model assessment of insulin resistance
HPLC	High performance liquid chromatography
HR	Hazard ratio
iAUC	Incremental area under the curve
IC_{50}	Dose needed to inhibited the activity by 50 %
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IQR	Interquartile range

KBS	Krebs buffer solution
KCH	King's College Hospital
KCL	King's College London
Ki	Inhibition constant
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LPH	Lactase phlorizin hydrolase
NDNS	National Diet and Nutrition Survey
NEFA	Non-esterified fatty acids
NO	Nitric oxide
OGTT	Oral glucose tolerance test
PA	Proanthocyanidins
PCA	Protocatechuic acid
PE	Phenol-Explorer
PI3K	Phosphoinositide 3-kinase
PPARs	Peroxisome proliferator-activated receptor
Pt	Phloretin
PWV	Pulse wave velocity
Pz	Phlorizin
RCT	Randomised controlled trial
RQUICKI	Revised quantitative insulin sensitivity check index
RR	Relative risk
SBP	Systolic blood pressure
SDS	Sodium dodecyl sulphate
SGLT1	Sodium-dependent glucose transporter 1
T2D	Type 2 diabetes
TAG	Triacylglycerol
Tmax	Time to reach maximum concentration
USDA	United Stated Department of Agriculture
WHO	World Health Organization

Chapter 1

General introduction

Parts of this General Introduction chapter have been published by in the Proceedings of the Nutrition Society: Castro-Acosta, M. L., *et al*. (2016). "Berries and anthocyanins: promising functional food ingredients with postprandial glycaemia-lowering effects." Proc Nutr Soc: (75), 342-355

Diet is a major determinant of risk of cardio-metabolic diseases, with dietary modification being the keystone to the prevention of chronic diseases. Epidemiological evidence and randomised controlled trials suggests that dietary patterns with high intakes of fruit and vegetables are associated with reduced risk of cardiovascular disease (CVD) and type 2 diabetes (T2D). Data from three prospective longitudinal cohort studies (n=66105 women, n=85104 women and n=36173 men) showed reduced risk of T2D for individual fruits, with hazard ratios (HR) of 0.74 (95 % CI 0.66, 0.83) for blueberries, 0.88 (95 % CI 0.83, 0.93) for grapes and raisins and 0.89 (95 % CI 0.79, 1.01) for prunes (Muraki *et al.*, 2013). In the Kuopio Ischaemic Heart Disease Risk Factor Study (cohort study n= 2332 men), intake of fruit, berries and vegetables showed a tendency for T2D reduced risk (HR 0.76 (95% CI: 0.57, 1.02; *P*-trend = 0.15)) when compared higher vs lower quartile (Mursu *et al.*, 2014). In the PREDIMED trial (n=7216 elderly men and women at high cardiovascular risk) consumption of nine or more servings/d of fruit and vegetables was associated with reduced risk of CVD (HR 0.60 (95% CI 0.40, 0.96, *P*=0.005)) compared with consumption of <5 servings/d (Buil-Cosiales *et al.*, 2016). Increasing fruit and vegetable consumption has been recommended for the prevention of chronic diseases, such as cardiovascular disease and type 2 diabetes (Bazzano, 2005). Fruits and vegetables are rich in fibre, micronutrients, and non-nutrient compounds, such as polyphenols, that may have beneficial health effects. This thesis will explore how polyphenols may contribute to some of these protective effects by regulating postprandial blood glucose concentrations.

1.1 Type 2 Diabetes

Type 2 diabetes (T2D) is a chronic disease characterised by an ineffective utilisation of glucose produced by two main defects in the body: diminished insulin sensitivity by muscle, liver and adipose tissue (insulin resistance) and inadequate insulin secretion by pancreatic β-cells (Oh *et al.*, 2012, WHO, 2016). Insulin is secreted from pancreatic β-cells in response to increased blood glucose concentrations, thereby stimulating glucose uptake and glycogen synthesis (insulin-mediated glucose metabolism). When insulin resistance is present, the pancreatic β-cells need to produce more insulin to compensate for impaired insulin action, resulting in hyperinsulinaemia. Insulin resistance also contributes to the raise of glucagon and catecholamines, which stimulate glycogenolysis and gluconeogenesis in liver increasing the release of glucose to blood circulation. However, the compensatory activity of pancreatic β-cells diminishes over the time making insulin production insufficient to maintain an adequate insulin-dependent

glucose uptake in skeletal muscle cells, resulting in a reduced glucose uptake and incremented blood glucose levels (hyperglycaemia) (Oh *et al.*, 2012, Papazafiropoulou *et al.*, 2012). Years of uncontrolled blood glucose may lead to micro and macrovascular complications, such as retinopathy, neuropathy, nephropathy, coronary heart disease, peripheral vascular disease or stroke. In addition, blindness, lower extremity amputations and end-stage renal disease account for other chronic complications (Banerjee and Saxena, 2012, ADA, 2013). Sustained hyperglycaemia is associated with oxidative stress, a condition where an imbalance between oxidant and antioxidant species leads to damage of cells or organs due to an oxidative burden (Sies *et al.*, 2005). Hyperglycaemia presenting in individuals with T2D can be improved or reversed by reduction of body weight, increasing physical activity and/or using oral glucose-lowering drugs, although if glucose level is not controlled by any of the aforementioned methods it is necessary to resort to insulin injection (ADA, 2013).

1.1.1 Risk factors for T2D

Type 2 diabetes represent 90% to 95% of the cases worldwide and according to the World Health Organization, 9 % of the world's population suffers from T2D and the number is predicted to reach exceptional levels (WHO, 2016). T2D is associated with age, obesity, history of impaired glucose tolerance, hypertension and dyslipidaemia as well as with unhealthy life habits (e.g. smoking, physical inactivity, unhealthy diet) (Banerjee and Saxena, 2012). Obesity is the primary cause of T2D, although it has been estimated that 10% of T2D patients are not overweight or obese (HSCIC, 2015), and most people with obesity do not develop T2D. Risk markers such as visceral fat and adipocytokine secretion, which are tightly associated with excess body weight, are likely to determine the progression to insulin resistance and eventually β-cell failure (Papazafiropoulou *et al.*, 2012). Other risk factors for the development of T2D are the two denominated prediabetic stages; impaired fasting glucose (IFG) and impaired glucose tolerance (IGT), which can evolve to T2D although not always inevitably (WHO, 2016). The glucose-stimulated insulin secretion has two phases, the first one (acute or early phase) imply a short and rapid period of insulin release to reduce the glucose production in liver, characteristic of fasted states. The second-phase (late phase) implies a more sustainable release of insulin to increase insulin-mediated glucose uptake and return glucose levels to fasting values. Deficiencies in the acute phase response and an exaggerated second-phase response are associated with IFG while disruptions in both phases response are present in individuals with IGT

(Papazafiropoulou *et al.*, 2012, Eikenberg *et al.*, 2016). Loss of the early phase response and deficiency in the second-phase response will elevate postprandial glycaemia.

Elevated postprandial glycaemia has been implicated in the development of T2D (Meyer *et al.*, 2000) and represents a risk factor that can easily be targeted by dietary modifications. The concentrations of glucose in the blood following a meal containing a known amount of carbohydrate represents the sum total of the rate of digestion and absorption of glucose in the gut, as well as the rate of uptake from the circulation into the cells for oxidation or storage. An exaggerated postprandial glycaemic response to a standard carbohydrate load is indicative of a reduction in insulin secretion or sensitivity. Reducing the rate of delivery of glucose to the bloodstream by manipulating the carbohydrate type and/or meal composition is one way in which these adverse metabolic profiles might be ameliorated. High dietary glycaemic index and glycaemic load independently increase risk of T2D (Barclay *et al.*, 2008) (relative risk 1.4 and 1.3 respectively). Frequent elevated excursions in postprandial glucose concentrations are thought to increase risk of T2D and CVD by inducing oxidative stress and glycation of proteins, as reviewed by Blaak *et al.* (Blaak *et al.*, 2012). Studies with acarbose (an inhibitor of α-glucosidase) show that reducing the rate of carbohydrate digestion can reduce the risk of progression to diabetes in participants with impaired glucose tolerance by 25% (Chiasson, 2006), suggesting that dietary strategies to control chronic postprandial hyperglycaemia by optimising the functionality of foods would strengthen efforts to reduce the risk for T2D. Dietary constituents that help to delay glucose absorption, e.g. fibre, polyphenols, following a carbohydrate-containing meal or beverage, would therefore also provide a potentially safe and effective approach to reducing risk of progression to T2D.

1.2 Dietary polyphenols and T2D

Epidemiological studies have shown a negative relationship between ingestion of polyphenols and cardiovascular disease (Hooper *et al.*, 2008, McCullough *et al.*, 2012, Jennings *et al.*, 2012, van Dam *et al.*, 2013, Cassidy *et al.*, 2013), cancer (Neuhouser, 2004, Arts and Hollman, 2005, Fink *et al.*, 2007, Yuan, 2011) and type 2 diabetes (Wedick *et al.*, 2012, Curtis *et al.*, 2012, van Dam *et al.*, 2013, Jacques *et al.*, 2013, Jennings *et al.*, 2014, Tresserra-Rimbau *et al.*, 2016). High intakes of subclasses of flavonoids have been associated with a reduced risk of T2D. A lower T2D risk was observed when intakes of flavonol and flavan-3-ol were greater in a USA population (n=2915 Framingham Offspring cohort) (Jacques *et al.*, 2013). Total flavonoid, flavonol and flavanol intakes with associated with reduced risk of T2D in The European Prospective Investigation into Cancer and Nutrition-InterAct case-cohort study (n=15258 EPIC-InterAct) (Zamora-Ros, 2013). In a more recent study in a case-cohort (n=3340 men and women) of the PREDIMED trial, flavonoid and proanthocyanidin intakes were inversely associated with diabetes risk (Tresserra-Rimbau *et al.*, 2016). Evidence from i*n vivo* and *in vitro* studies support the epidemiological data. Human clinical trials have provided indication of a decreased postprandial glucose after intake of polyphenol-rich food followed by a high carbohydrate meal. Decreased postprandial hyperglycaemia has been shown following consumption of berries (Törrönen *et al.*, 2012a, Hoggard *et al.*, 2013) and apple juice (Johnston *et al.*, 2002). Lower postprandial glucose levels have also been demonstrated *in vivo* by individual compounds of polyphenol subclasses such as flavan-3-ols (Gutierrez-Salmean *et al.*, 2014), flavonols (Hussain *et al.*, 2012), isoflavones (Bhathena and Velasquez, 2002) and phenolic acids (van Dijk *et al.*, 2009). *In vitro* studies support the *in vivo* data by showing potential inhibitory effects on carbohydrate digestion and absorption by polyphenols, mechanisms proposed are inhibition of digestive enzymes α-amylase and α-glucosidases and glucose transporters in the intestinal tract (Hanhineva *et al.*, 2010, Babu *et al.*, 2013). I*n vitro* inhibition of digestive enzymes activities has been demonstrated by anthocyanins (Akkarachiyasit *et al.*, 2010), proanthocyanidins (McDougall, 2008), flavan-3-ols and phenolic acids (Hanhineva *et al.*, 2010) among others. In addition i*n vitro* inhibition of glucose transporters has been showed by flavan-3-ols and flavones (Kwon *et al.*, 2007), anthocyanins, flavanones and isoflavones (Kottra and Daniel, 2007).

1.3 Classification and main food sources of polyphenols

Polyphenols are a large group of plant-based, bioactive compounds widely distributed in foods of plant origin, and form an important part of the human diet. Fruits, vegetables and beverages produced of plants such as tea, coffee, red wine and fruit juices represent the main dietary sources of polyphenols although dry legumes, cereals and chocolate also contribute to the total consumption (Manach *et al.*, 2004). More than 500 different polyphenols have been identified in food (Perez-Jimenez *et al.*, 2010b), modifications in the chemical structure modify their biological efficacy, bioavailability and permit its classification into classes and subclasses (Williamson and Manach, 2005). Polyphenols are commonly classified in four major groups; flavonoids, phenolic acids, lignans and stilbenes. The flavonoid group, usually the major component of dietary polyphenols, can be further broken down into subclasses including anthocyanins, flavanols, flavanones, flavones, flavonols and isoflavones. Phenolic acids represent the second component of dietary polyphenols followed by lignans and stilbenes. Average intakes of polyphenols vary between countries and populations, and are commonly related to socio-demographics and life style factors. In European countries total polyphenol intakes vary from 584 mg/d to 1786 mg/d and main food sources are non-alcoholic beverages such as coffee, tea and fruit juices, fruits and vegetables, and alcoholic beverages as wine (Zamora-Ros *et al.*, 2015). **Table 1.1** presents polyphenol classifications, with the most common subclasses consumed in a normal diet and their richest food sources. Some subclasses' richest food sources are foods with very low intake frequency and/or very low quantity intakes e.g. hydroxycinnamic acids and dried peppermint, however the main food sources in daily diets are usually foods with a lower content but that are consumed more frequently and in a higher quantity e.g. hydroxycinnamic acids and coffee.

Table 1.1 Classification of most common polyphenols according to Phenol-Explorer database

Polyphenols Classes and subclasses	**Main food sources**
Flavonoids	
Anthocyanins	Black elderberry, black chokeberry, blackcurrant , blueberry
Dihydrochalcones	Oregano, plum/prune juice, apple
Flavanols	Cocoa powder, dark chocolate, broad bean, black/green tea, apple
Flavanones	Peppermint dried, oregano, grapefruit juice, orange juice
Flavones	Celery seed, peppermint dried, oregano, celery leaves
Flavonols	Cappers, saffron, oregano, red onion, spinach, shallot, chokeberry
Isoflavones	Soy flour, soy paste, soybean, soy tempeh, soy yogurt/milk
Phenolic acids	
Hydroxybenzoic acids	Chestnut, cloves, pomegranate juice, raspberry, cranberry
Hydroxycinnamic acids	Peppermint dried, common verbena, rosemary dried, spearmint
Lignans	
Matairesinol	Sesame seed, linseed, sunflower seed, kale, raisin
Secoisolariciresinol	Linseed, cashew nut, kale, kiwi, peanut
Stilbenes	
Resveratrol	Red wine, lingonberry, red currant, cranberry, bilberry
Other polyphenols	
Alkylmethoxyphenols	Coffee, rapeseed, beer
Alkylphenols	Breakfast cereals, rye wholegrain flour, common wheat, rye bread
Hydroxybenzaldehydes	Walnut, red wine, fennel, vinegar, oat whole grain flour
Hydroxycoumarins	Chinese cinnamon, white wine, beer, sherry, cocoa powder
Phenolic terpenes	Rosemary, common sage
Tyrosols	Black/green olive, olive oil, sherry, red wine, vinegar

Adapted from database Phenol-Explorer (Neveu *et al*., 2010, Rothwell *et al*., 2012, Rothwell *et al*., 2013)

Polyphenols have in common the presence of one or more phenolic ring in their backbone structure. Flavonoids share a general structure consisting of two phenolic rings bound together by three atoms of carbon that form an oxygenated heterocycle ($C_6C_3C_6$), labelled as rings A, B and C, respectively (Manach *et al.*, 2004). They are divided into different subclasses on function of the degree of hydroxylation, methoxylation, prenylation or glycosylation (Gonzales *et al.*, 2015). Phenolic acids share the basic structure of a phenolic group and a carboxylic acid (C_6C_1); they are classified into different subclasses in function of the degree of hydroxylation, conjugation with different groups and glycosylation (Del Rio *et al.*, 2013). The main phenolics acids in food are derivatives of hydroxybenzoic and hydroxycinnamic acids, the latter are mainly conjugated with tartaric acid and quinic acid forming a group denominated chlorogenic acids (Manach *et al.*, 2004, Stalmach *et al.*, 2012). The common chemical structure of lignans is formed by two phenyl groups united by two propane units ($C_6C_3C_3C_6$), lignans more commonly found in food are

secoisolariciresinol and matairesinol (Manach *et al.*, 2004). Stilbenes can occur in a vast diversity of forms in the plant kingdom, although their molecular skeleton consist of two phenolic rings united by an ethane unit ($C_6C_2C_6$), variation in structure is due to the degree of polymerisation on the backbone structure, the pattern of construction of oligomers and the different units present in it. Stilbenes can be classified into two groups, monomeric and oligomeric stilbenes, and themselves can be divided into subgroups. The largest subgroup of the oligomeric stilbenes is resveratrol (Shen *et al.*, 2009). See **Figure 1.1** for chemical structures of the four main classes of polyphenols.

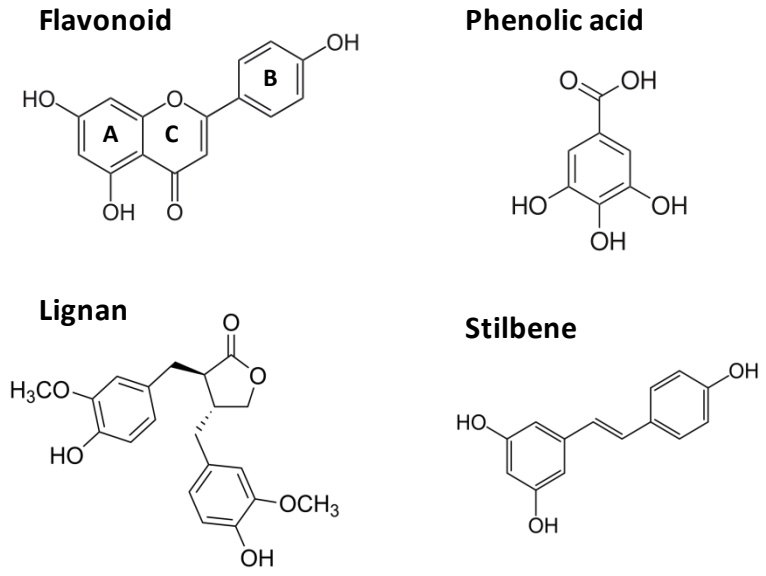


Figure 1.1 Chemical structures of the four main classes of polyphenols

1.4 Bioavailability of polyphenols

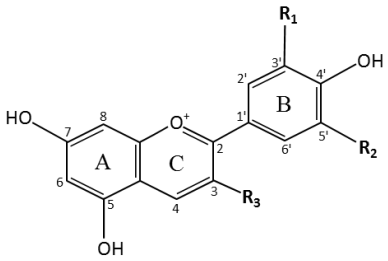
The bioavailability of polyphenols is affected by factors such as chemical structure, food matrix, food processing, enzymatic activity in mouth, small intestine and liver as well as gut microbiota. Structural variations as hydroxylation, glycosylation, polymerisation, etc. can affect polyphenol absorption, distribution, metabolism and excretion in the body. After consumption polyphenols are absorbed in the intestinal tract into enterocytes, although absorption might start in stomach for some flavonoids and stilbenes. Flavonoids, lignans, and stilbenes are usually found in food as glycosides, with a sugar moiety added to the backbone structure, although some flavanols are mainly present as aglycones. Aglycones can be absorbed directly into enterocytes but glycosides have to be hydrolysed in order to be absorbed. In enterocytes sulfation,

glucoronidation and/or methylation reactions prepare the metabolites before entering portal circulation in their way to liver. Once in liver several of those metabolites are catabolised, reactions of oxidation, reduction or hydrolysis introduce or eliminate hydroxyl and amino groups, making them accessible for the next series of reactions. Conjugation with glucuronic acid, glutathione, or glycine and sulfation reactions produce metabolites soluble in water that can be excreted in urine. After metabolism in liver, flavonoid metabolites might be directed to plasma for their subsequent dissemination within the body via bloodstream or redirected to small intestine via bile (Manach *et al.*, 2004, Piskula *et al.*, 2012), once redirected to small intestine metabolites might be reabsorbed or reach colon to be hydrolysed by the microbiota and reabsorbed into the circulatory system (Vitaglione *et al.*, 2012). High molecular weight metabolites, such as proanthocyanidins, can be partially digested in small intestine however most of them will continue their way to colon and undergo metabolism by microbiota, and a proportion of these metabolites are reabsorbed before transport to the liver (Crozier *et al.*, 2012). Phenolic acids may cross passively through the intestinal epithelium (Stalmach *et al.*, 2012), a minor part of the lignans is absorbed in small intestine, with the unabsorbed portion continuing its way to the large intestine where intestinal microbiota metabolise lignans into enterodiol and enterolactone (Manach *et al.*, 2004) prior to absorption (Patel *et al.*, 2012). Stilbenes undergo similar process to flavonoids, the portion of dietary polyphenols not absorbed in small intestine can reach colon and be metabolised by microbiota for their subsequent absorption or excretion in faeces (Manach *et al.*, 2004). Considering their different chemical and biological properties, it is important to study dietary polyphenols as individual or as similar compounds (classes and subclasses) rather than as an entire homogeneous group (Perez-Jimenez *et al.*, 2010b).

1.5 Berries and anthocyanins

Anthocyanins are a subclass of the flavonoid group and are mostly responsible for the bright and deep colours associated with certain fruits and vegetables such as grapes, berries, cherries, aubergine and red onion (Manach *et al.*, 2005). The six most abundant anthocyanin aglycones (anthocyanidins) are malvidin, petunidin, delphinidin, peonidin, pelargonidin and cyanidin. These anthocyanidins share a structure of 2-benzene rings (A and B rings) united by a heterocyclic ring (C ring) (**Fig. 1.2**). As suggested by their name, aglycones are not bound to a sugar molecule. However, polyphenolic compounds mainly exist as *O*-glycosides in plants, where a sugar moiety resides most often at

position 3 of carbon ring C (**Fig. 1.2**). Commonly linked sugars include glucose, galactose, arabinose and rutinose. For example, blackcurrants are high in delphinidin-O-3-rutinoside and cynanidin-O-3-rutinoside (Rothwell *et al.*, 2013, Neveu *et al.*, 2010, Rothwell *et al.*, 2012). Aside from sugars, anthocyanins may also be found acylated to aromatic and aliphatic acids. The variety of ways in which anthocyanins might be glycosylated or acylated has led to reports of up to 650 varieties of anthocyanins identified so far in flowers, fruits, vegetables and other plant material (Andersen and Jordheim, 2010).



Anthocyanidin	R_1	R_2	R_3	Anthocyanin	R_3
Pelargonidin	-H	-H	-OH	Pelargonidin-3-O-glucoside	-O-glucose
Cyanidin	-OH	-H	-OH	Cyanidin-3-O-rutinoside	-O-rutinose
Delphinidin	-OH	-OH	-OH	Delphinidin-3-O-rutinoside	-O-rutinose
Peonidin	$-OCH_3$	-H	-OH	Peonidin-3-O-glucoside	-O-glucose
Petunidin	$-OCH_3$	-OH	-OH	Petunidin-3-O-glucoside	-O-glucose
Malvidin	$-OCH_3$	$-OCH_3$	-OH	Malvidin-3-O-glucoside	-O-glucose

Figure 1.2 Structure of the most common anthocyanidins and anthocyanins found in berries

1.5.1 Main sources and dietary intake

Dietary intakes of anthocyanins are derived from a relatively narrow range of foods. The total anthocyanin content varies widely from 0.28 to 1480 mg/100g in both fruit and vegetables. The main sources in the human diet are berries with blue, purple and orange/red pigments. Berries with the largest concentrations are elderberries, chokeberries, blackcurrants and blueberries with estimated contents in the range of 160 to 1300 mg per 100 g fresh weight (Koponen *et al.*, 2007, Wu *et al.*, 2006, Rothwell *et al.*, 2013, Bhagwat, 2013, Manach *et al.*, 2004, Fernandes *et al.*, 2013) (**Table 1.2**). Mean estimates from food frequency questionnaires (FFQ) and 24-hour recalls (24-HR) in adult populations from different countries vary from 0.04 (Lako J, 2006) to 215 mg/d (Kuhnau, 1976), but with the majority or reports ranging between 18 and 43 mg/d (Zamora-Ros *et al.*, 2011b, Jennings *et al.*, 2012, Cassidy *et al.*, 2011, Wu *et al.*, 2006, Perez-Jimenez *et al.*, 2011, Li *et al.*, 2013, Johannot and Somerset, 2007).

Table 1.2 Average content of polyphenols in berries (mg/100 g fresh weight)

Common name	Scientific name	Total phenolics (Folin assay)	Estimated total ACNs	Major ACN
American cranberry	*Vaccinium macrocarpon* Aiton	315	32[a]/50[b]	peonidin-3-galactoside
Bilberry or whortleberry*	*Vaccinium myrtillus*	525	299[a]/38[b]	delphinidin-3-glucose; delphinidin-3-galactoside
Black chokeberry	*Aronia melanocarpa*	1752	444[a]/878[b]	cyanidin-3-galactoside
Black elderberry	*Sambucus nigra*	1950	1317[b]	cyanidin-3-glucoside
Blackberry	*Rubus sp.*	569	147[a]/173[b]	cyanidin-3-glucoside
Blackcurrant	*Ribes nigrum*	821	225[a]/592[b]	delphinidin-3-rutinoside
Gooseberry	*Ribes uva-crispa*	470	14[a]/9[b]	cyanidin-3-glucoside
Grapes (black)	*Vitis vinifera*	185	72[b]	malvidin-3-glucoside
Highbush blueberry	*Vaccinium corymbosum*	223	164[a]/134[b]	delphinidin-3-galactoside; malvidin-3-galactoside
Lingonberry	*Vaccinium vitis-idaea*	652	45[a]/60[b]	cyanidin-3-galactoside
Red raspberry	*Rubus idaeus*	155	44[a]/73[b]	cyanidin-3-sophoroside
Redcurrant	*Ribes rubrum*	448	26[a]/35[b]	cyanidin-3-xylosyl-rutinoside
Strawberry	*Fragaria sp.*	289	73[b]	pelargonidin-3-glucoside

ACNs, anthocyanins, [a]total estimate from pH differential method by spectrophotometry; [b]sum of individual anthocyanin concentrations estimated by chromatography (may underestimate due to missing compounds). Values will vary widely depending on cultivar, environmental factors, method of analysis, particularly underestimation due to unidentified/unquantified compounds. All values are averages based on the Phenol-Explorer database www.phenol-explorer.eu, excepting bilberry*(Chu *et al.*, 2011).

FFQs may underestimate true anthocyanidin intakes since questions are not specific to individual fruits and averaged values are applied to groups of foods that may vary widely in their anthocyanin contents, for example in the EPIC-Norfolk FFQ "Strawberries, raspberries, kiwi fruit" are grouped together in one category to indicate frequency of consumption, and other anthocyanin-rich foods as blueberries are not mentioned in the questionnaire. Food diaries may provide a more accurate representation of intake, but estimates represent short-term intakes rather than habitual consumption patterns, which could be particularly misleading for seasonally available foods like berries. Research groups have created and validated FFQ to estimate dietary flavonoid intake in different populations (Somerset and Papier, 2014, Theodoratou *et al.*, 2007), which should provide more reliable intake estimations for specific populations, although they remain unavoidably susceptible to bias due to self-reporting errors, portion size quantification and estimation errors resulting from the lack of data on polyphenol content in food (Zamora-Ros *et al.*, 2014). At present, the most common databases employed to assess flavonoid intakes are the USDA (Bhagwat, 2013, Bhagwat, 2008, Nutrient Data Laboratory, 2004) and Phenol-Explorer (Rothwell *et al.*,

2013), which provide information on the content of 35 flavonoids in 506 food items and 502 polyphenols (of the 4 classes) in 459 food items, respectively. The USDA database expressed flavonoid content as aglycone equivalents exclusively while Phenol Explorer database expressed polyphenol content as aglycones, glycosides or esterified metabolites and also includes retention factors to calculate changes in content due to cooking process. Although the Phenol Explorer database offers a wealth of detailed data on the polyphenol composition of foods, it might still be considered a work in progress when considering the broad diversity of polyphenols in food and the remaining gaps in the food analytical literature. New techniques for intake estimation have been examined; these include innovative technologies for measuring dietary intakes in epidemiological studies (Illner *et al.*, 2012), as well as biomarker approaches. The use of metabolomic techniques to analyse phenolic metabolites in urine or plasma has a promising role in epidemiological studies (Manach *et al.*, 2009, Edmands *et al.*, 2015). Although current estimates of dietary anthocyanins intakes are limited, epidemiological studies suggest that higher consumption rates of berries and anthocyanins are associated with beneficial effects on risk factors related to vascular function and T2D.

1.5.2 Epidemiological studies

Prospective and cross-sectional studies in different populations have investigated associations between berry consumption and the risk of T2D, providing some support for a potentially protective effect arising from increased berry intakes.

Prospective cohort and cross-sectional studies

An inverse association between high consumption of berries and risk of T2D was observed in a Finnish cohort study (n=10,054 men and women), with a hazard ratio (HR) of 0.74 (95% CI: 0.58, 0.95) when comparing highest and lowest quartiles (Knekt *et al.*, 2002). The Kuopio Ischaemic Heart Disease Risk Factor Study in Finnish middle-aged men (n=2,682) reported that consumption of >59.7 g of berries per day compared to <1.3 g lowered risk of T2D (mean follow up 19 y), with a multivariable-adjusted HR of 0.65 (95% CI: 0.49, 0.88). Importantly, total fruit or vegetable consumption had no statistically significant fully adjusted association with T2D risk, possibly signifying a more potent role of berries in modulation of risk (Mursu *et al.*, 2014).

High intakes of anthocyanins and anthocyanin-containing foods were significantly associated with a lower risk for T2D in US men and women (n=199,980, 3 cohorts),

with a pooled HR of 0.85 (95% CI: 0.80, 0.91) for the highest quintile of anthocyanidin intakes compared with the lowest. Cyanidin exhibited the strongest effect on T2D risk; HR: 0.79 (95% CI: 0.72, 0.85), followed by malvidin, delphinidin, peonidin and petunidin. Blueberry intakes were most closely negatively associated with T2D risk (HR 0.77, 95% CI 0.68, 0.87), as well as apple/pear intakes, followed by strawberry intakes (Muraki *et al.*, 2013, Wedick *et al.*, 2012). In contrast, although lower T2D risk was observed when intakes of flavonol and flavan-3-ol were greater (n=2915 Framingham Offspring cohort, USA), no associations with anthocyanin intake were detected (Jacques *et al.*, 2013). Furthermore, contrary to the aforementioned studies, there was no association between total flavonoid or anthocyanin intake and risk of T2D in the Iowa Women's Health Study prospective cohort (n=35,816 postmenopausal women, USA) (Nettleton *et al.*, 2006).

Recently, a high intake of anthocyanins was found to be associated with lower insulin resistance (HOMA-IR) in a cross-sectional study using the Twins UK registry (n=1997 women, UK). Women reporting higher intakes of anthocyanidins by FFQ (Q1: 3.5 mg/day; Q5: 40 mg/day) had lower HOMA-IR scores and lower fasting serum insulin levels following adjustment for BMI, age, smoking, physical activity, diet, menopausal status and medication. More specifically, higher intakes of delphinidin, malvidin and petunidin were associated with lower HOMA-IR and insulin levels (Jennings *et al.*, 2014). This supports longitudinal observations of T2D risk and suggests that anthocyanins may reduce T2D risk by modulating insulin resistance independently of BMI and other major dietary factors. Inconsistent findings from longitudinal studies might be due to limitations and errors inherent to dietary intake methodology. Overall, there are sufficient epidemiological data to support a likely relationship between greater intakes of berries, anthocyanin-rich foods and anthocyanins, and reduced risk of T2D in adult populations.

1.5.3 Bioavailability of anthocyanins

Bioavailability of anthocyanins was formerly believed to be low (<2 %) (Del Rio *et al.*, 2013), with levels in plasma varying from 1 to 592 nM following the consumption of an anthocyanin-rich meal (Kay, 2006) and up to mM values in the gut lumen (Williamson, 2013). However, it was demonstrated using a stable isotopically labelled anthocyanin that bioavailability may not be lower than other flavonoids; bioavailability of cyanidin-3-glucoside was estimated to be at least 12 % calculated from ^{13}C recovery in urine and

breath (Czank *et al.*, 2013). Anthocyanin metabolites excreted in urine correspond to 15 % of total intake when consuming 300 g of raspberries in a low polyphenol diet (Ludwig *et al.*, 2015). Human studies have shown that the time to reach maximum concentrations in plasma varies between 0.5-4 h (Kay, 2006, Del Rio *et al.*, 2013). This is consistent with evidence showing that anthocyanins may be partly absorbed in the stomach before reaching the small intestine (Talavera *et al.*, 2003, Fernandes *et al.*, 2012).

After ingestion, anthocyanins appear to permeate the stomach mucosa; proposed mechanisms include a bilitranslocase carrier and a saturable transporter, GLUT1 (Passamonti *et al.*, 2003, Oliveira *et al.*, 2015, Fernandes *et al.*, 2012). Further down the gastrointestinal tract, in the small intestine many flavonoid glycosides undergo hydrolysis of the sugar moiety by the membrane-bound enzyme, lactase phlorizin hydrolase (LPH) with subsequent passive diffusion of the aglycone into the enterocyte. However, some anthocyanin glycosides, such as cyanidin-3-glucoside and cyanidin-3-galactoside, have shown resistance to LPH (Fang, 2014, Nemeth *et al.*, 2003). In fact, anthocyanins that are absorbed in the small intestine are more likely to be taken up into enterocytes intact, their metabolites then being formed in the small intestine after absorption (de Ferrars *et al.*, 2013, Fang, 2014). Any deglycosylation within the gut lumen primarily occurs in the colon due to the action of gut microbiota, as reviewed by Fang, 2014 (Fang, 2014).

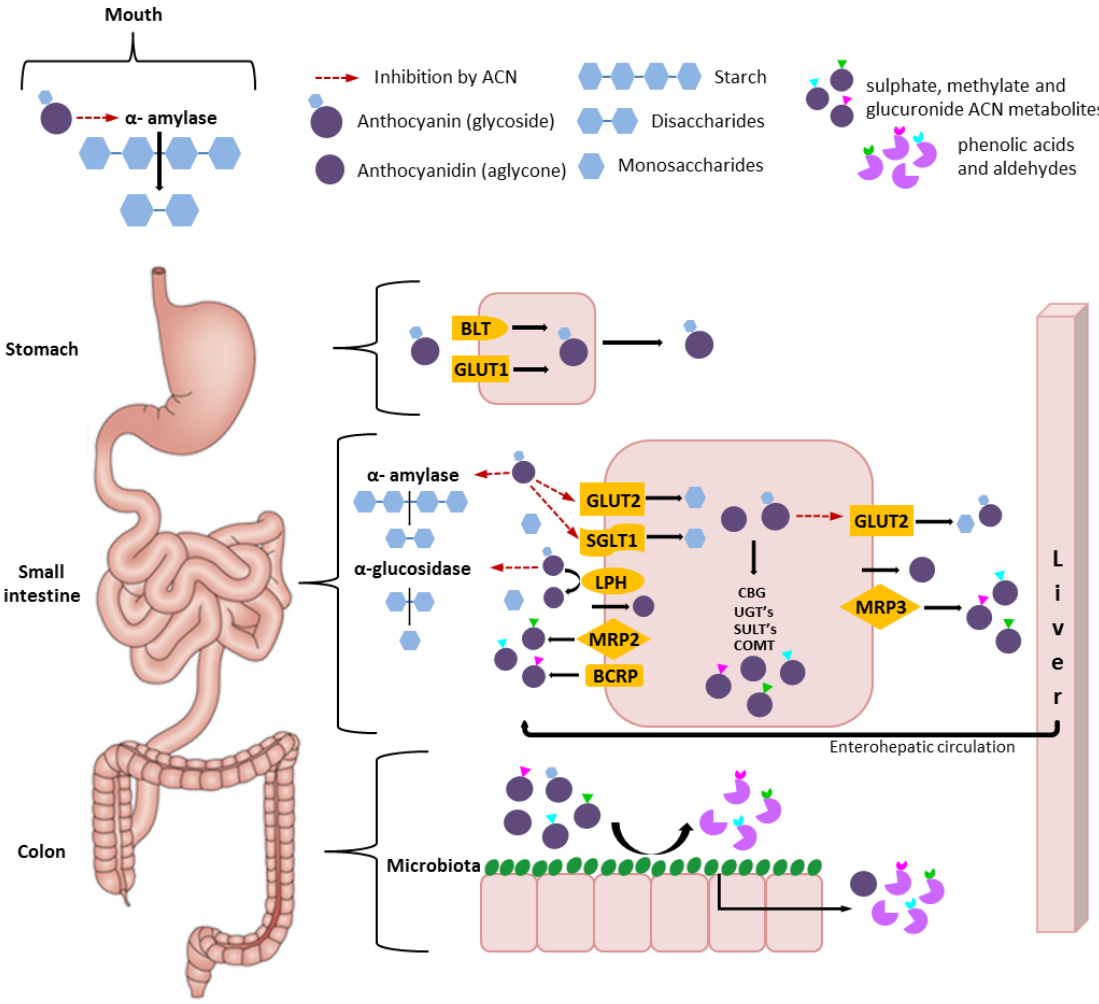
Early studies suggested a role for the sodium-dependent glucose transporter 1 (SGLT1) for the absorption of glycosides (Day *et al.*, 2003), but SGLT1 expressed in *Xenopus* oocytes does not transport flavonoid glycosides (Kottra and Daniel, 2007). In enterocytes, intact glycosides may undergo the action of the cytosolic-β-glucosidase, which cleaves the sugar moiety and releases the free aglycone (Day *et al.*, 1998); aglycones then undergo phase II metabolism by sulfotransferases, methyltransferases and glucoronyltransferases, forming sulphated, methylated and glucuronidated metabolites (Del Rio *et al.*, 2013). Efflux of metabolites into the small intestine may occur via transporters inserted into the luminal membrane, such as multidrug resistance protein 2 and breast cancer resistance protein (van de Wetering *et al.*, 2009, Chen *et al.*, 2014). Phase II metabolites reach portal circulation via active transporters inserted in the basolateral membrane, such as multidrug resistance protein 3 (van de Wetering *et al.*, 2009, Chen *et al.*, 2014), studies have also suggested the action of the glucose

transporter GLUT2 (Day *et al.*, 1998, Williamson *et al.*, 2000, Manzano and Williamson, 2010).

Once in the portal circulation, metabolites can reach the liver and undergo additional phase II metabolism before entering the systemic circulation (Kay, 2006), from where they are directed to several organs and tissues (e.g. adipose tissue, heart, eyes, cerebrum, kidneys) to exert their biological effects or to be metabolised and eliminated in urine (Fernandes *et al.*, Rodriguez-Mateos *et al.*, 2014). Anthocyanin metabolites could be directed to the enterohepatic circulation for their subsequent excretion into the small intestine via bile for reabsorption or make their way to the large intestine to be transformed by microbiota and then reabsorbed or eliminated in faeces (Fernandes *et al.*, Talavera *et al.*, 2003). Unabsorbed anthocyanins reaching the large intestine may be converted to other metabolites by resident colonic bacteria, followed by absorption or excretion in the faeces (Gonzalez-Barrio *et al.*, 2010, Gonzalez-Barrio *et al.*, 2011, Del Rio *et al.*, 2013). Microbiota can degrade anthocyanins to phenolic acids and aldehydes by splitting the C-ring and modifying the remaining A and B-ring (Rodriguez-Mateos *et al.*, 2014). Some of the main metabolites of microbiota degradation are gallic acid, vanillic acid, homovanilic acid, protocatechuic acid (PCA), syringic acid and 4-hydroxybenzoic acid (Vitaglione *et al.*, 2007, Azzini *et al.*, 2010, Fleschhut *et al.*, 2006, Forester and Waterhouse, 2008, de Ferrars *et al.*, 2013). Despite knowledge of the high rate of anthocyanin degradation by gut microbiota there is still not consensus about the proportion absorbed into systemic circulation (Fang, 2014, Czank *et al.*, 2013, de Ferrars *et al.*, 2013). See **Fig. 1.3** for proposed mechanism of anthocyanin absorption.

Studies showing higher bioavailability of anthocyanins have been able to detect a broader spectrum of metabolites in blood and urine samples. For example, a study published in 2007 reported only the recovery of cyanidin-3-glucoside and PCA in plasma following ingestion of one litre of blood orange juice (Vitaglione *et al.*, 2007). However, de Ferrars *et al. (*de Ferrars *et al., 2013)* reported 28 total metabolites, 17 phenolics and 11 anthocyanin conjugates in urine following consumption of an elderberry extract (500 mg mixed anthocyanins) consisted mostly of cyanidin glycosides, while plasma analysis discovered 17 phenolics and four anthocyanin conjugates. Urine samples demonstrated high amounts of vanillic acid and conjugates and were more abundant in anthocyanin conjugates, while plasma was highest in 4-hydroxybenzaldehyde and protocatechuic acid-sulfate (de Ferrars *et al.*, 2013). Isolated and ^{13}C-labelled cyanidin-3-glucoside was traced in healthy males, demonstrating a

total of 25 different metabolites, including a range of cyanidin glucuronides and methyl compounds as well as aldehydes and phase II PCA conjugates (Czank *et al.*, 2013). The metabolism of raspberry anthocyanins produced 18 detectable compounds in urine, including cyanidin-3-O-glucoside, peonidin-3-O-glucoside and 16 phenolic metabolites, while in plasma nine anthocyanins metabolites were quantified including glucuronides and sulphated compounds (Ludwig *et al.*, 2015). Furthermore, not all metabolites were recovered, which is in concordance with more recent data that identified a total of 36 metabolites in serum, urine and faecal samples following ingestion of 500 mg labelled cyanidin-3-glucoside (de Ferrars *et al.*, 2014). These studies raise the possibility that the health effects associated with berries and their anthocyanins may be in part attributable to metabolites of parent anthocyanin compounds. It is now apparent that parent anthocyanins maintain a relatively short half-life, whereas their metabolites, which includes phase I and II compounds, are active for longer and reach higher maximum concentrations (de Ferrars *et al.*, 2014).



ACN, anthocyanin; BLT, bilitranslocase; GLUT1, glucose transporter type 1; GLUT2, glucose transporter type 2; SGLT1; sodium-dependent glucose transporter 1; LPH, lactase phlorizin hydrolase; MRP2, multridrug resistance protein 2; BCRP, breast cancer resistance protein; CBG, cytosolic β-glucosidase; UGT's, glucoronyltransferases; SULT's, sulfotransferases; COMT, catechol-O-methyltransferase; MRP3, multridrug resistance protein 3.

Figure 1.3 Metabolism of carbohydrates and effects of anthocyanins on enzymes and glucose transporters. Adapted by permission in part from MacMillan Publisher Ltd: Nature Reviews Immunology (Mowat and Agace, 2014), copyright 2015. After ingestion, anthocyanins appear to permeate the stomach mucosa; possibly via the BLT carrier and GLUT1. In the small intestine, anthocyanins can inhibit the action of digestive enzymes and reduce glucose transport, the latter mechanism possibly by competition with glucose for GLUT2 and SGLT1. Anthocyanins that are absorbed in the small intestine are likely to be taken into enterocytes intact, but may also be hydrolysed by LPH to their aglycone before absorption. In enterocytes, intact glycosides may undergo the action of cytosolic-β-glucosidase, which cleaves the sugar moiety and release the free aglycone. Their glucuronidated, sulphated and methylated metabolites can then be formed in the small intestine after absorption. Anthocyanins that reach the lower small intestine and colon may be deglycosylated and further metabolised to phenolic acis by gut microbiota prior to absorption.

1.5.4 Human studies

Research investigating the role of polyphenols in carbohydrate digestion and absorption in humans is largely dominated by randomised, placebo-controlled trials, considered the gold standard in scientific research. However, the varying types of berries and berry combinations, the methods of administering the treatments and the specifics of each study design bring a substantial amount of variation. Details of these studies are outlined in **Table 1.3**.

Edirisinghe *et al*. (Edirisinghe *et al.*, 2011) tested the effects of a strawberry extract milk-based drink on plasma glucose and serum insulin levels. Although no changes in glucose were observed, insulin levels were significantly higher in the placebo arm. The lack of effect on glucose levels could be explained by the presence of milk proteins, which may compete with intestinally-derived proteins (enzymes, transporters expressed on the luminal surface) for polyphenol binding (Gonzales *et al.*, 2015, Charlton *et al.*, 2002). Postprandial glucose and insulin responses were reduced in overweight subjects who consumed 0.47 g of encapsulated bilberry extract (equivalent to a 50 g serving of bilberries) alongside 75 g Polycal liquid as an oral glucose tolerance test (Hoggard *et al.*, 2013). Interestingly, the most significant effects were observed at later time points (120, 150 and 180 min), in contrast with other studies (Törrönen *et al.*, 2012a, Törrönen *et al.*, 2012c).

Lingonberry and blackcurrant purées significantly lowered plasma glucose at 15 and 30 mins, and increased plasma glucose after 60 min relative to control, post-ingestion of 35 g sucrose, and blackcurrant nectar showed comparable results (Törrönen *et al.*, 2012a). Similar studies using a mixed berry purée of blackcurrant, strawberries, cranberries and bilberries demonstrated consistent time-dependent glucose and insulin responses to 35 g sucrose (reduced 15-45 min, increased at ~90 min), together with a borderline increase in plasma concentrations of the gut peptide glucagon-like peptide-1 (GLP-1) in the early postprandial phase (Törrönen *et al.*, 2010, Törrönen *et al.*, 2012c). Interestingly, these experiments, all by the same group, consistently demonstrate that berries inhibit the early phase of postprandial glycaemia, when sucrose is being hydrolysed by α-glucosidase into glucose and fructose, and glucose is being rapidly absorbed from the upper small intestine, whereas there is a compensatory increase in the later postprandial phase relative to control, suggesting a more protracted glycaemic response to sucrose ingestion when consumed with berries.

More recently, the same group reported that a blackcurrant, strawberry, bilberry and cranberry purée reduced postprandial glycaemia 0-30 min in response to white wheat bread consumption (50 g starch) by 32 % (Törrönen *et al.*, 2013). On the other hand, a study investigating the effect of blueberries and raspberries found no differences in postprandial glycaemia compared to control, although the authors suggested that there may have been inadequate mastication of the whole berries and therefore limited release of free polyphenols from cell walls (Clegg *et al.*, 2011). Additional studies investigating the effects of anthocyanins on glycaemic responses to solid and liquid mixed meals containing starches and/or sucrose will further elucidate the potential effects of berry polyphenols on metabolic response.

The above mentioned studies investigate the acute response to the administration of anthocyanins; however, there may also be a longer-term effect as a consequence of daily consumption. Obese men and women administered a blueberry smoothie (668 mg anthocyanins; 1462 mg total phenolics) twice daily for 6 weeks demonstrated a positive effect on insulin resistance compared to a nutritionally matched blueberry-free smoothie (Stull *et al.*, 2010). Subjects followed an *ad libitum* diet, suggesting the addition of this amount of blueberries, around two cups fresh blueberries per day, even without dietary changes may influence the current state of insulin resistance, although it is possible that other dietary changes were made in a motivated study population.

Overall, the evidence base to date indicates an inhibitory effect of berry extracts or purées in the initial postprandial glycaemic response, suggesting that berry components such as anthocyanins and proanthocyanidins might be acting in the gut as a "brake" on the rate of glucose absorption, but not the total amount. However, the majority of the study designs involve administering whole berries or berry purées, making it difficult to pinpoint the relative effects of the polyphenols versus other components such as fibre, which might delay the rate of glucose absorption by slowing down gastric emptying. It is impossible to distinguish the relative contributions of the berry anthocyanins, proanthocyanidins, ellagitannins, flavonols, and phenolic acids from the human studies published to date. *In vitro* experiments using Caco-2 cells and controlled expression of glucose transporters in *Xenopus* oocytes may help to shed light on these questions in order to optimise the efficacy of mixed berry extracts intended for nutraceutical or functional food applications. Further randomised controlled trials using alternative study designs may also address the relative contributions of anthocyanins compared to other berry polyphenols, for example comparing high anthocyanin blackcurrants with

low anthocyanin greencurrants, or using powdered berry extracts concentrated in polyphenol content and containing minimal concentrations of other nutrients/non-nutrients.

Table 1.3 Summary of randomised controlled acute and chronic dietary intervention trials using berry meals

References	*n* (sex), status, country	Subjects age		Design	Dietary intervention	Food/ACN (dose/day)	Effects	Quality assessment
		Mean	SD					
(Stull *et al.*, 2010)	32 (M+F), insulin resistant, USA	51	3	DB P; chronic (6 wk)	freeze-dried BB powder	668 mg ACN	- stimulation of IS	+
(Törrönen *et al.*, 2010)	12 (M+F), healthy, Finland	54	15	SB CO; acute	puree of BC, BIB, CB and SB	150 g	- ↓plasma glucose in early stages of post prandial glucose curve	Æ
(Edirisinghe *et al.*, 2011)	24 (M+F), overweight, USA	51	15	SB CO; acute	freeze-dried SB powder	82 mg ACN	- insulin ↓ at 60 and 180 min post meal - improved IS in post prandial state	+
(Clegg *et al.*, 2011)	12 (M+F), healthy, Finland	33	13	CO; acute	RB- or BB-containing pancakes	100 g berries	- no effect on postprandial glucose response	+
(Törrönen *et al.*, 2012c)	12 (M+F), healthy, Finland	58	11	SB CO; acute	puree of BC, BIB, CB and SB	150 g	- glucose curve normalised	+
(Törrönen *et al.*, 2012a)	20 (F), healthy or overweight, Finland	57	10	SB CO; acute	BB puree and nectar, LB puree and nectar	150 g	- normalised postprandial glucose curve by puree and less-so by nectar	+
(Hoggard *et al.*, 2013)	8 (M), T2D, UK	62	5	DB CO; acute	BIB extract	169 mg ACN	- 18 % ↓plasma glucose iAUC following OGTT	+

Table 1.3 continued

References	*n* (sex), status, country	Subjects age Mean	SD	Design	Dietary intervention	Food/ACN (dose/day)	Effects	Quality assessment
(Törrönen *et al*., 2013)								
-Study 1	15 (F), healthy, Finland	48	14	SB CO; acute	puree of SB, BIB or LB	150 g	- SB puree may improve glycaemic response	+
-Study 2	13 (F), healthy, Finland	50	12	SB CO; acute	puree of RB, CLB or CHB	150 g	- no significant effects on blood glucose	+
-Study 3	20 (F), healthy, Finland	57	12	SB CO; acute	puree of SB, BIB, CB + BC	150 g	- ↓early postprandial hyperglycaemia; - ↑ glycaemic profile	+
(Nyambe-Silavwe and Williamson, 2016)	16 (NS), healthy, UK	26	4	SB CO; acute	freeze-dried AP, BKB, BC, SB powder + GT powder	40.5/81 g	- 27 % and 49 % ↓plasma glucose iAUC - 47 % ↓ insulin iAUC	+

Quality assessment was based on the method recommended by the American Dietetic Association (ADA, 2009). Studies were assessed and given one of the following three scores: Minus (-), indicating that the study scored poorly on at least 6 out of 10 points; Neutral (Æ), indicating that the study was not exceptionally strong (scores were poor on points relating to validity/reliability of outcomes, whether the intervention was described in enough detail, whether selection of study participants was free from bias, and whether study groups were comparable and methods of randomisation was described and unbiased); and Plus (+), indicating that the study was of very high quality, i.e. scores were high on the previous 4 points and also high on at least one other point. ACN, anthocyanins; M, males; F, females; USA, United States of America; SB CO, single-blind cross-over; SB, strawberry; T2D, type 2 diabetes mellitus; UK, United Kingdom; DB CO, double-blind cross-over; BIB, bilberry; iAUC, incremental area under the curve; OGTT, oral glucose tolerance test; BB, blueberry; LB, lingonberry; BC, blackcurrant; CB, cranberry; RB, raspberry, CLB, cloudberry; CHB, chokeberry; CO, cross-over. DB P, double-blind parallel, ; IS, insulin sensitivity; NS, non-specified; AP, apple peel; BKB, blackberrry; GT, green tea.

1.5.5 Berries and anthocyanins; modulation of glucose metabolism (*In vitro* studies)

Consumption of anthocyanin-rich foods have been associated with beneficial effects on metabolic biomarkers in humans, including postprandial concentrations of glucose, insulin, free fatty acids and gastrointestinal hormones such as glucose-dependent insulinotropic polypeptide (GIP) and GLP-1 (Törrönen *et al.*, 2012a, Törrönen *et al.*, 2010, Törrönen *et al.*, 2012c, Johnston *et al.*, 2005, Johnston *et al.*, 2002). There is increasing evidence for a potential role for dietary anthocyanins in glucose homeostasis, but there is a lack of understanding of the mechanisms by which these effects are played out. In order to elucidate the mechanisms different extracts and individual compounds have been tested using *in vitro* experiments. Enzymatic studies suggest that anthocyanins may inhibit digestive enzymes such as salivary and pancreatic α-amylases and α-glucosidases; sucrase and maltase (**Figure 1.3**). Studies using Caco-2 cells, as a model of the small intestine, and *Xenopus laevis* oocytes, expressing glucose transporters, show sugar uptake inhibition by anthocyanin extracts and individual compounds (**Figure 1.3**).

Carbohydrates digestion and absorption

Carbohydrate digestion begins in the mouth by α-amylases that hydrolyse α(1,4)-glycosidic bonds of polysaccharides (e.g. starch), which are broken down into smaller peptides, amylose and amylopectin (Englyst *et al.*, 2007). In the small intestine, additional pancreatic α-amylases are secreted. Alpha-glucosidases act on sucrose and maltose, generating monomers of glucose and fructose that are absorbable by brush border cells of the small intestine. Glucose may be transported by SGLT1and GLUT2 at the apical membrane, the latter of which is primarily functional during high luminal glucose concentrations (Wright *et al.*, 2007, Kellett *et al.*, 2008). Higher free glucose in the gut lumen may influence the net uptake of glucose, contributing to a higher release of glucose into the bloodstream. Therefore, the ability to slow the rate of carbohydrate digestion and the release of free glucose may be important in managing postprandial hyperglycaemia.

1.5.5.1 Digestive enzymes: *in vitro* studies

Anthocyanins are highly bioactive molecules, but defining their bioactivities *in vivo* can present many challenges. As previously mentioned, anthocyanins are thought to reach millimolar concentrations within the gut lumen and intestinal tissues, although only nanomolar concentrations are present in the blood stream (Fang, 2014). The relatively higher concentrations in the gut tissues may provide sufficient potency for the effects observed

within *in vitro* studies, which are discussed further below. Many cellular models testing inhibition of digestive enzymes and intestinal transporter proteins by anthocyanins have found IC_{50} values within the range of micromolar concentrations, which are well within a physiologically feasible range. Strawberry extracts from various species of Brazilian strawberry significantly inhibited α-glucosidase activity up to 70 % in a dose-dependent manner (Da Silva *et al.*, 2008). Although these effects were marked, it is difficult to pinpoint whether enzyme activity inhibition was attributable to specific anthocyanins or other polyphenols in the strawberry extract. Therefore, many *in vitro* studies focus on testing individual anthocyanins, as described below. The results of these studies are also displayed in **Table 1.4**.

One of the first studies investigating the effects of cyanidin-3-galactoside, high in blueberries, lingonberries and cranberries, showed inhibition of both sucrase and maltase enzyme activity. Furthermore, kinetic analysis suggested both competitive and non-competitive inhibition, meaning cyanidin-3-galactoside may interact directly at the active site of the enzyme, inhibiting substrate binding, or at another site (Adisakwattana *et al.*, 2009). Comparable results were observed by the same authors for cyanidin-3-rutinoside, which is found in particularly concentrated amounts in blackcurrants (Adisakwattana *et al.*, 2011a). Similarly, sucrase was inhibited to a larger extent than maltase, and cyanidin-3-rutinoside was significantly more potent compared to cyanidin-3-galactoside, which might be related to the disaccharide structure of rutinose (Adisakwattana *et al.*, 2011a). Aglycone cyanidin also shows inhibition of sucrase activity, although to a much lesser extent than its glucosides, while cyanidin-3,5-diglucoside showed relatively no inhibition (Akkarachiyasit *et al.*, 2010) and cyanidin-3-rutinoside show inhibition of α-amylase as well in a non-competitive manner (Akkarachiyasit *et al.*, 2011). These data highlight the potent effects of cyanidin glycosides on carbohydrate-digesting enzymes within the gut, and suggest that cyanidin glycoside-containing berry species, such as blackcurrant, blackberry and lingonberry (Rothwell *et al.*, 2013), might have particularly potent postprandial glycaemia-lowering effects.

Separate extracts of strawberry, raspberry, blueberry and blackcurrant showed dose-dependent inhibition of α-amylase, with strawberry and raspberry (also rich in hydrolysable tannins, ellagitannins) demonstrating the most significant effects (McDougall *et al.*, 2005). Anthocyanins present in high amounts in raspberries and strawberries include cyanidin and pelargonidin glycosides, however they may also contain significant amounts of phenolic acids and other flavonoids (Rothwell *et al.*, 2013). Alternatively, blackcurrant and blueberry extracts were more potent α-glucosidase inhibitors in comparison to the other two extracts.

By separating the anthocyanin-containing portion of the extract from the whole raspberry extract, McDougall *et al* (McDougall *et al.*, 2005) demonstrated that the inhibitory effects on α-amylase are largely mediated by non-anthocyanins, while α-glucosidase activity is modulated by the anthocyanin-containing portion. In fact, in a separate study it was found that the inhibitory effects of rowanberries on α-amylase are primarily carried out by proanthocyanidins, a flavonoid subclass constituted of dimers, oligomers or polymers of catechins or epicatechins linked together, also known as condensed tannins (Manach *et al.*, 2004), by their protein-binding activity (Grussu *et al.*, 2011). This was further exemplified by the low inhibition of α-glucosidase by a proanthocyanidin-rich rowanberry extract, signifying the specific importance of this group of compounds for α-amylase inhibition (Boath *et al.*, 2012). Both red and yellow raspberries significantly influenced α-amylase activity, suggesting that synergism between proanthocyanidins, which are more concentrated in yellow raspberries compared to red, anthocyanins, which are much higher in red raspberries, and other compounds such as ellagitannins, flavonols and hydroxycinnamic acids may occur to effect α-amylase inhibition (Grussu *et al.*, 2011). More recently, the same authors provided significant evidence of anthocyanin-rich blackcurrant extract inhibition of α-glucosidase *in vitro (Boath et al., 2012)*. However, no synergistic effects were apparent after combining the extracts (Boath *et al.*, 2012). As this particular rowanberry extract was low in anthocyanins, it suggests they are not the sole compounds contributing to these effects, as observed for proanthocyanidins and α-amylase inhibition.

Acarbose is a competitive inhibitor of maltase and sucrase in the digestive tract and is a drug used in the management of T2D. European regulations suggest doses between 25-200 mg three times daily, depending on severity of disease (Fischer *et al.*, 1998). Gastrointestinal side effects associated with acarbose have limited the success of the drug (Aoki *et al.*, 2010). Administration of certain polyphenols may pose synergistic effects on sucrase and maltase activity (Adisakwattana *et al.*, 2009, Adisakwattana *et al.*, 2011a, Akkarachiyasit *et al.*, 2010, Akkarachiyasit *et al.*, 2011) depending on doses of polyphenol and acarbose (Adisakwattana *et al.*, 2009). Together these studies suggest that acarbose acts via different mechanisms than some polyphenols, providing the synergistic inhibition observed, and may give insight into strategies to lower doses in acarbose treatment to diminish side-effects associated with the drug.

Table 1.4 *In vitro* evidence for berries and anthocyanins inhibition at digestion enzymes

Reference	Enzyme source	Extract/ACN	Dose	IC_{50}	Enzyme	Effect
(Adisakwattana *et al*., 2009)	RIAP	C3Gal	1 mM	sucrase: 0.5 mM	sucrase maltase	- inhibition of α-glucosidase and synergism with acarbose - mixed type inhibition
(Adisakwattana *et al*., 2011b)	RIAP	C3R	0.1, 0.5 and 1.0 mM	sucrase: 250.2 µM maltase: 2.3 mM	sucrase maltase	- bigger inhibition on sucrase than maltase
(Akkarachiyasit *et al*., 2010)	RIAP PP α-amylase	cyanidin; C3Gal; C3G; C3,5-dG.	NS	sucrase: 1.42 mM (cyanidin); 0.97 mM (C3G); 0.50 mM (C3Gl) α-amylase: 0.38 mM (cyanidin); 0.30 mM (C3G)	sucrase maltase α-amylase	- α-amylase and sucrase inhibited by cyanidin glucosides - C3,5-dG shows no inhibition - synergistic effects of acarbose with C3G, C-3,5-dG and C3Gal
(Akkarachiyasit *et al*., 2011)	PP α-amylase	C3R	0.1-1.0 mM	α-amylase: 24.4 µM	α-amylase	- dose-dependent inhibition of α-amylase - synergism with acarbose
(Boath *et al*., 2012)	RIAP	PA-rich BC and RWB extract	10-40 µg/mL	BC: 20 µg/mL GAE RWB: 30 µg/mL GAE	α-glucosidase	- dose-dependent inhibition
(Da Silva *et al*., 2008)	PP α-amylase RI α-glucosidase	Brazilian SB extracts	10, 25 and 50 mg/mL	NS	α-glucosidase α-amylase	- dose-dependent inhibition of α-glucosidase, up to 70 % - lower inhibition of α-amylase

Table 1.4 continued

Reference	Enzyme source	Extract/ACN	Dose	IC_{50}	Enzyme	Effect
(Grussu *et al*., 2011)	PP α -amylase	fractionated RWB extract - yellow RB extract - red RB extract	NS	whole extract: epicatechin-containing PA: 5 µg/mL (8.4 µM) - 16.5 µg/mL GAE - 13.5 µg/mL GAE	α-amylase	- whole extract and epicatechin-containing PA inhibited α-amylase
(McDougall *et al*., 2005)	RIAP; PP α-amylase	extracts of SB, RB, BB and BC	50-500 µg of phenols	NS	α-amylase; α-glucosidase	- α-amylase inhibited by non-ACN fraction; SB + RB most efficient - α-glucosidase inhibited by ACN; BC + BB dose-dependent inhibition
(Nyambe-Silavwe and Williamson, 2016)	Human salivary α-amylase*	Sugar-free extracts of BKB, BC and SB	0-4 mg of powder/ml	BKB: 1.2 and 1.6 mg/ml BC: 1.5 and 1.7 mg/ml SB: 2.5 and 3.9 mg/ml	α-amylase	- α-amylase inhibited by powder extracts of BKB, BC and SB at physiological concentrations

ACN, anthocyanin; RIAP, rat intestinal acetone powder; C3Gal, NS, non-specified; C3Gal, cyanidin-3-galactoside; C3R, cyanidin-3- rutinoside; PP, porcine pancreatic; C3G, cyanidin-3-glucoside; C-3,5-dG, cyanidin-3,5-diglucoside; PA, proanthocyanidin; BC, blackcurrant; RWB, rowanberry; SB, strawberry; RI, rat intestinal; RB, raspberry; GAE, gallic acid equivalent; BB, blueberry; BKB, blackberry.

* using amylose and amylopectin as substrates

1.5.5.2 Sugar uptake: *in vitro* studies

Following the release of glucose from sucrose and starch by digestive enzymes, glucose absorption may be further disrupted by interactions between berry anthocyanins and intestinal sugar transporters. The human cell line Caco-2, has been widely used as an *in vitro* model of the small intestine, the cell line obtained from a human colon adenocarcinoma, under culture conditions develop as a cell monolayer with characteristics of a mature enterocyte. Caco-2 cell system expresses the enzymes sucrase, maltase and lactase, along with the transporters GLUT1, GLUT2, GLUT3, GLUT5 and SGLT1 on its apical border (Sambuy *et al.*, 2005).

Johnston *et al.* tested the effect of polyphenols on glucose transport in the Caco-2 cell line. Although no berry-specific anthocyanins were tested, it was one of the first studies suggesting competitive inhibition of SGLT1 and inhibition of GLUTs in a cellular model of the human intestinal lining (Johnston *et al.*, 2005). Manzano and Williamson (Manzano and Williamson, 2010) monitored the rate luminal glucose uptake and of basolateral GLUT-mediated glucose transport, showing that strawberry extract is able to hinder translocation to a much larger extent within sodium-free conditions (GLUT-mediated), although transport was also inhibited during sodium-dependent conditions (SGLT1 and GLUTs), suggesting that the inhibition of GLUTs was greater than the inhibition of SGLT1. Strawberry extract (50-400 mg/ml) showed inhibition of total (SGLT1 and GLUTs) apical glucose uptake and basal transport on the Caco-2 cells *in vitro* model, with an IC_{50}= 324 mg/ml, a high concentration compared to physiological levels. Although the specific strawberry compounds responsible for these effects were not clear, HPLC analysis showed the extract was relatively high in pelargonidin-3-glucoside, which showed inhibition of glucose transport into and across the Caco-2 cell (IC_{50}= 802 μM) (Manzano and Williamson, 2010). These results suggest that strawberry compounds may influence glucose transport across intestinal cells, thereby modulating the rate of glucose flux into the bloodstream. More recently, Alzaid *et al.* (Alzaid *et al.*, 2013) demonstrated that acute exposure of Caco-2 cells to cyanidin, cyanidin-3-glucoside and cyanidin-3-rutinoside (100 μM each), significantly reduced total and facilitated (GLUT-mediated) glucose transport. Whole mixed berry extract (4 mM) also significantly inhibited uptake of glucose in this model (Alzaid *et al.*, 2013).

Although the exact mechanisms for anthocyanin absorption are not clearly identified, glucose transporters GLUT2 and SGLT1 may play an important role. An i*n vitro* study

using Caco-2 cells treated with cyanidin-3-glucoside showed decreased absorption of the anthocyanin when specific inhibitors of GLUT2 and SGLT1 were added compared to without inhibitors; the same effect was observed in cells with decreased expression of GLU2 and SGLT1, suggesting the involvement of both transporters in the absorption of cyanidin-3-glucoside (Zou *et al.*, 2014). Furthermore, expression of GLUT2 in the apical side of Caco-2 cells was decreased at the same time as glucose uptake was decreased following cyanidin-3-glucoside treatment (Faria *et al.*, 2009). Competition between glucose and anthocyanins for glucose transporters may represent a potential mechanism for inhibition of postprandial glycaemia.

Interestingly, longer-term exposure (16 h) of berry extracts (4 mM) to Caco-2 cells showed an inhibitory effect on both GLUT2 and SGLT1 expression, which was further exemplified at the protein level for GLUT2. However, the 16 h exposure showed minimal effects on total glucose uptake, with only facilitated (GLUT-mediated transport) uptake demonstrating a statistically significant reduction (Alzaid *et al.*, 2013). Caco-2 cell model pre-treated for 96 h with anthocyanin extract (200 μg/ml) increased the expression of GLUT2 but not SGLT1 and GLUT5. Acute glucose transport, mediated by GLUT2 was decreased in cells pre-treated with anthocyanin extract and malvidin-3-glucoside (200 μg/ml), pre-treatment with malvidin (200 μg/ml) showed no inhibitory effect (Faria *et al.*, 2009). In summary, the evidence for opposing results on glycoside and aglycone treatments suggest that the sugar moiety may interfere on GLUT2-mediated glucose transport and upregulation of GLUT2 gene expression may be relevant for longer term glycaemic control.

In vitro studies using *Xenopus* oocytes to express either SGLT1 or GLUT2 under controlled conditions have shown an inhibitory effect of polyphenols and anthocyanins on glucose uptake. *Xenopus laevis* oocytes are resilient cells that are easy to handle in laboratory studies. The expression system in oocytes has gained popularity due to their high transcriptional capacity and their ability to express multi-subunit proteins correctly assembled, post-translationally modified and correctly orientated following the injection of exogenous RNA or DNA (Gurdon *et al.*, 1971, Sigel and Minier, 2005, Bianchi and Driscoll, 2006). Pelargonidin and pelargonidin-3-glucoside inhibit glucose absorption (1 mM), with IC_{50}= 1.34 and 2.47 mM respectively, in oocytes expressing SGLT1 (Kottra and Daniel, 2007). In a separate *in vitro* study using *Xenopus* oocytes expressing GLUT2, Kwon *et al.* (Kwon *et al.*, 2007) were not able to detect an inhibitory effect on glucose uptake (10 mM) when testing delphinidin and cyanidin (0-300 μM), although

their glycosidic forms were not tested and other flavonoids present in berries, such as quercetin, were observed to inhibit GLUT2 mediated glucose transport.

Overall, experiments using oocytes expressing individual sugar transporters and cultured intestinal cells have shown that berry extracts and individual anthocyanins may interfere with glucose transport from the gut lumen into the enterocyte, and also across the basolateral membrane into the blood. This may occur via inhibition of GLUT2 under postprandial glycaemic conditions, since this transporter is believed to be functionally important under conditions of high luminal glucose concentrations (Zheng *et al.*, 2012). However, inhibition of SGLT1 activity may also play a significant role in berry polyphenol-mediated control of glucose homeostasis since SGLT1 is instrumental in the regulation of GIP and GLP-1 secretion and upregulation of GLUT2 (Gorboulev *et al.*, 2012). Extracts of berries used in the different studies may contain a range of different classes of polyphenols, and further *in vitro* studies testing individual anthocyanins are needed in order to pinpoint exact mechanisms for these intestinal transport effects.

1.6 Apple polyphenols

Polyphenol content in fruit and vegetables is influenced by several factors as cultivar, ripeness and environmental factors as well as storage and processing conditions. Simple processing and cooking methods (freezing, cutting, slicing, heating, etc.) can alter the polyphenol content, e.g. a peeled apple differs in polyphenol content from an unpeeled one (Manach *et al.*, 2004). Despite the likely variability in polyphenol content within food categories, databases on food average polyphenol content have been developed (Bhagwat, 2013, Neveu *et al.*, 2010) and are commonly used to estimate polyphenols content in food and dietary intake. The main polyphenol subclasses in apple (*Malus domestica*) are represent by flavonoids followed by phenolic acids (**Table 1.5**). Apple is particularly rich in flavanols monomers (flavan-3-ols) and polymers (proanthocyanidins), although flavonols and phenolic acids are also present in considerable quantities. Characteristic phenolic compounds of apple are the dihydrochalcones, phloretin and its glycoside phlorizin; both compounds are found in peel (exocarp), flesh (mesocarp), seeds and stems (pedicel) of apple although higher concentrations are contained in peel than in flesh (Tsao *et al.*, 2003). Phloretin and phlorizin have showed a wide variety of beneficial effects on *in vitro* and *in vivo* studies, including control of blood glucose and antioxidant capacity (de Oliveira, 2016). The diversity of polyphenols contained in apple and it worldwide availability had made it an excellent food source of polyphenols in human diet.

Table 1.5 Major polyphenols contained in unpeeled raw dessert apple

Polyphenols	**mg/100 g FW[1]**
Flavonoids[2]	145.9
Anthocyanins	0.9
Cyanidin-3-O-galactoside	0.8 ± 0.9
Dihydrochalcones	5.4
Phloretin 2'-O-xylosyl-glucoside	2.6 ± 2.1
Phlorizin (phloretin-2'-O-glucoside)	2.7 ± 1.9
Flavanols	24.2
(+)-Catechin	1.2 ± 0.8
(-)-Epicatechin	8.4 ± 3.7
Procyanidin dimer B2	14.6 ± 9.2
Proanthocyanidins (PA)[3]	108.6
01mers	7.2 ± 3.5
02-03 mers	19.8
04-06 mers	26.5 ± 5.2
07-10 mers	22.5 ± 4.2
Polymers (> 10 mers)	32.7 ± 7.0
Flavonols	6.9
Quercetin	0.1 ± 0.1
Quercetin-3-O-arabinoside	1.4 ± 1.1
Quercetin-3-O-galactoside	2.4 ± 1.2
Quercetin-3-O-glucoside	0.6 ± 0.8
Quercetin-3-O-rhamnoside	1.3 ± 1.6
Quercetin-3-O-rutinoside	0.2 ± 0.1
Quercetin-3-O-xyloside	0.8 ± 0.6
Phenolic acids[2]	19.0
Hydroxybenzoic acids	1.1
Hydroxycinnamic acids	17.9
4-p-coumaroylquinic acid	2.3 ± 1.9
5-caffeoylquinic acid (Chlorogenic acid)	13.4 ± 11.3
5-p-coumaroylquinic acid	1.1 ± 0.1
Total polyphenols[4]	200.9 ± 104.8

All values are mean ± SD
[1]FW fresh weight, all values are averages based on the Phenol-Explorer database www.phenol-explorer.eu
[2] Analysed by chromatography
[3] Analysed by normal phase HPLC
[4] Analysed by Folin-Ciocalteau assay

1.6.1 Dietary intake

Dietary intake of flavonoids and phenolics acids estimated from FFQ and 24-HR in adult populations from different European countries vary from 200 to 1000 mg/d for flavonoids and from 250 to 900 mg/d for phenolic acids. The study showed the main food sources of flavonoids and phenolic acids for Mediterranean and non-Mediterranean countries and for a UK health-conscious group, "apples and pears" accounted for 22, 13 and 5% of the flavonoid, and 4, 2 and 2% of phenolic acid intake, respectively. The main consumed individual flavonoids were proanthocyanidins polymers (>10 mers) and proanthocyanidins oligomers (4-6 mers) while the most consumed phenolic acids were 5-caffeoylquinic acid (chlorogenic acid), 4-caffeoylquinic acid and 3-caffeoylquinic acid (Zamora-Ros *et al.*, 2015).

The same study also provided estimates of intakes of the main polyphenol subclasses contained in apple in a UK general population. Intake of flavan-3-ols monomers and proanthocyanidins in men and women were 213.5 ± 5.4 mg/d and 178.6 ± 4.5 and 217.1 ± 12.8 mg/d and 172.9 ± 10.5, respectively. Main food sources of flavan-3-ols monomers were tea (51%), followed by "apples and pears" (12%), whereas "apples and pears" were the main food source of proanthocyanidins (22% of intake) followed by tea (15%) (Knaze *et al.*, 2012). In the same population flavonols intake were 51.0 ± 2.2 mg/d and 42.4 ± 1.8 mg/d in men and women respectively. In the same study but in a UK health-conscious population, flavonol intakes were 54.9 ± 4.1 mg/d and 50.1 ± 3.1 mg/d in men and women, respectively (Zamora-Ros *et al.*, 2011a) and "apples and pears" were the main food source of procyanidin B2, proanthocyanidin oligomers (4-10 mers) and proanthocyanidin polymers (>10 mers) and the third of chlorogenic acid (Zamora-Ros *et al.*, 2015). All together these data revealed that apples represent one of the main food sources of flavonoids and phenolic acids.

1.6.2 Epidemiological studies

Epidemiological studies have shown an association between polyphenols intakes and reduced risk of T2D. Especial focus on flavonoid food sources and flavonoid subclasses has shown a reduced risk of T2D at higher apple, and its major flavonoid (flavanol and flavonol), intakes. A case-cohort study in a Finish population (n=10054 men and women) reported that consumption of >47 g of apple per day compared to no consumption (0 g) was inversely associated with occurrence of T2D. Although not significant association was found, a tendency in reduced risk of T2D was related with

higher quercetin intake with a relative risk (RR) of 0.81(95% CI: 0.64, 1.02) when comparing highest and lowest quartiles (Knekt *et al.*, 2002). In the Women's Health Study, a randomised controlled trial in a US population (n= 38,018) consumption of $\geq$1 apple per day compared with no apple intake showed a significant reduced risk of T2D with a multivariate-adjusted RR of 0.72 (95% CI: 0.56, 0.92) (Song *et al.*, 2005). Recently in an elderly people at high cardiovascular risk-cohort (n=3340 men and women) of the PREDIMED trial, flavonoid and proanthocyanidin intakes were inversely associated with diabetes risk with a HR of 0.67 (95% CI: 0.48, 0.93) and 0.75 (0.54, 1.04), respectively, when compared highest to lowest tertile (Tresserra-Rimbau *et al.*, 2016). In the Framingham Heart Study Offspring Cohort (n=2915) flavonol intake was associated with a lower incidence of T2D with a multivariable-adjusted HR of 0.74 (95% CI: 0.61, 0.90) when compared highest to lowest percentile (Jacques *et al.*, 2013). In addition an inverse association between risk of T2D and high consumption of the flavan-3-ols monomers; catechin and epicatechin and proanthocyanidins; dimers and trimers was observed in a European case-cohort study population (n=26,088 men and women). Catechin and epicatechin showed a HR of 0.86 (95% CI: 0.75, 0.99) and 0.84 (0.69, 1.04) respectively, when compared highest and lowest quintiles. Proanthocyanidins dimers and trimers showed a HR of 0.81 (95% CI: 0.71, 0.92) and 0.91 (0.80, 1.04), respectively (Zamora-Ros *et al.*, 2013). Epidemiological data suggests that apple and apple flavonoids may reduce T2D risk, although associations must be considered cautiously due to limitations on dietary assessment methodology, such as questionnaires not validated for polyphenol intakes and errors in self-reported intakes. Moreover higher intakes of fruit and/or flavonoids are related to indicators of a healthier lifestyle, therefore some confounders might be present in the models even after adjustment.

1.6.3 Bioavailability of apple polyphenols

The main requisite for polyphenols in order to exert any effect *in vivo* is their absorption from the gastrointestinal tract into bloodstream in order to reach target organ and tissues. Bioavailability of flavonoids and phenolic acids in apple and apple-derived food depends of several factors as food matrix, food processing, enzymatic activity in mouth, small intestine and liver and gut microbiota. See section **1.5.3** for further details on flavonoid metabolism.

In a study on subjects with colectomy with terminal ileostomy, consuming one litre of cloudy apple juice (250 mg of polyphenols) only 33% of the polyphenols ingested were collected in the ileostomy bags after 6-8 h, which in normal conditions would reach the colon. Results suggested that the remaining 67% of the polyphenols were absorbed into enterocytes. Maximum excretion in ileostomy extract was reached at 2 h post consumption (Kahle *et al.*, 2006). Of the total polyphenols collected (8 h) only 14% of the parental compounds determined in the apple juice were recovered. Different compound such as caffeic acid, procyanidins B, (+)-catechin, phlorizin, quercetin-3-O-glucoside, quercetin-3-O-galactoside, and quercetin-3-O-xyloside were not detected in the collected fluid, suggesting that these compounds were absorbed or metabolised in small intestine. The aglycone phloretin, although not present in the apple juice, was quantified in the ileostomy extract with a maximum excretion between 2-4 h and the phloretin glucuronide was detected after 1 h with a maximum concentration at 2 h, indicating that phlorizin was hydrolysed before absorption (Kahle *et al.*, 2005). A subsequent study by the same group identified in 24 h urine samples 90-95 % of the polyphenols consumed. Approximately 20% of the polyphenols consumed were found in the forms of hydroxylated phenolic and hippuric acids (Kahle *et al.*, 2011). Results suggest a high bioavailability of apple polyphenols when consumed in juice. *In vivo* studies have tested the effect of the food matrix on individual apple polyphenols' bioavailability. A randomised controlled trial testing the bioavailability of epicatechin (241 μmol), when contained in drinks supplemented with apple extract and in an apple purée, showed that epicatechin metabolism was faster when provided in the supplemented drink, Tmax 0.9 vs 1.7 h, respectively. Plasma concentration area under the curve (AUC 0–24 h), absorption and urinary excretion of epicatechin were also higher after consumption of epicatechin contained in drink compared with apple purée (Hollands *et al.*, 2013). Results suggest that the apple purée matrix affected the bioavailability of epicatechin in small intestine. Other phenolic compounds present in apple have showed different metabolism absorption rate when consumed as pure compound vs contained in apple juice. Supplementation with one gram of chlorogenic acid showed absorption rate of 33% (Olthof *et al.*, 2003), while consumption of 113 mg of chlorogenic acid contained in apple juice showed an absorption rate of 90% (Kahle *et al.*, 2005). A latter study by Kahle's group analysing serum and 24 h urine samples showed maximum absorption of chlorogenic acid at 0.7 h and recovery of 95-99% of apple-derived phenolic acids, indicating a high metabolism of phenolic acids contained in apple juice (Kahle *et al.*, 2011).

1.6.4 Human studies on postprandial glycaemia

Reports of clinical trials have shown that consumption of apple juice and apple extracts reduce significantly plasma glucose concentrations in early postprandial response. Individual polyphenols contained in high quantities in apple or apple derived food have also shown a lowering effect on plasma glucose response following the consumption of a high carbohydrate meal.

In previous reports a cloudy apple juice containing 26 mg of phlorizin, 25 g glucose and 30.7 g fructose significantly lowered plasma glucose in human volunteers compared to a control drink, as demonstrated by a lower glucose incremental area under the curve (iAUC) 0-30 and 30-90 min (Johnston *et al.*, 2002). Furthermore, a dose of 2.8 g of an apple extract that appeared to be derived from apple pomace, containing a very large dose (448 mg) of phlorizin, reduced plasma glucose iAUC between baseline and 15, 30 and 45 min in healthy human subjects following a 75 g glucose load (Schulze *et al.*, 2014), and 25 g of unripe apple extract, derived from apple pomace, containing 315 mg of phlorizin reduced postprandial glucose response in healthy volunteers following by a 50 g glucose load (Makarova *et al.*, 2015). The use of individual apple polyphenols in high and acute doses has also proved a hyperglycaemic effect. In a randomised clinical study 400 mg of quercetin reduced the plasma glucose AUC 0-180 min after a maltose load (2 g/Kg) but not after a glucose load (100 g) in T2D patients (Hussain *et al.*, 2012). In a separate clinical study one gram of chlorogenic acid reduced plasma glucose and insulin concentrations after 15 min of an oral glucose tolerance test (OGTT) (van Dijk *et al.*, 2009). Epicatechin, dosed at one mg per kg of body weight, consumed prior to an oral metabolic tolerance test (39 g of carbohydrates, 9 g of proteins and 6 g of lipid) decreased postprandial glycaemia at 2 h in normal and overweight subjects (Gutierrez-Salmean *et al.*, 2014). All together these findings suggest that apple polyphenols might have a protective effect on glucose response by decreasing plasma glucose levels after a high carbohydrate load.

1.6.5 Effect of apple and apple polyphenols on carbohydrate metabolism

Potential mechanisms for hypoglycaemic effect of apple polyphenols include inhibition of digestive enzymes activity, decrease of intestinal glucose absorption, and stimulation of insulin secretion and protection of pancreatic β-cells.

1.6.5.1 Digestive enzymes: *in vitro* studies

During digestion of carbohydrates digestive enzymes, α-amylase and α-glucosidase play a central role degrading polysaccharides and oligosaccharides into shorter-chain oligosaccharides, disaccharides and ultimately, monosaccharides.

In vitro studies have shown inhibitory effects on the activity of digestive enzymes α-amylase and α-glucosidase (maltase and sucrase) by extracts from pulp and peel of different apple varieties. Concentrations from 10 to 500 µl of the extracts showed high α-amylase and α-glucosidase inhibitory activities in a dose-response manner. Peel extracts showed a higher α-glucosidase and a low α-amylase inhibitory activity when compared with pulp extracts while pulp extracts proved a higher α-amylase and low α-glucosidase inhibitory activity. A positive correlation between α-glucosidase inhibitory activity and total phenolics in extracts was observed, peel extracts demonstrated higher total phenolic content than pulp extracts. However no correlation between α-amylase inhibitory activity and total phenolic content was observed (Barbosa *et al.*, 2010).

Apple polyphenols compounds of the flavonoid and phenolic acid subclasses have showed inhibitory effect on α-amylase and α-glucosidases activity. Flavan-3-ols catechin and epicatechin inhibited maltase activity with IC_{50} of 1.4 and 1.8 mM, respectively and sucrase activity with IC_{50} of 2.5 and 2.0 mM, respectively (Ishikawa *et al.*, 2007), showing a marker effect on maltase activity. The flavonol quercetin showed inhibition of α-glucosidase; maltase and sucrase activity with IC_{50} of 0.7 and 1.5 mM, respectively (Ishikawa *et al.*, 2007). In a separate study quercetin also proved to be an inhibitor of rat small intestine maltase and porcine pancreatic α-amylase with an inhibition of 28 and 50 % by quercetin 0.5 mM, respectively (Tadera *et al.*, 2006). Later studies showed quercetin as an effective α-amylase activity inhibitor with and IC_{50} of 21 µM, modelling studies suggested flavonols act by binding to the active site of the enzyme (Lo Piparo *et al.*, 2008), and α-glucosidases activity inhibitor; maltase and sucrase (Pereira *et al.*, 2011). Chlorogenic acid (5-caffeoyl quinic acid) showed inhibitory activity on α-glucosidases; sucrase and maltase action with IC_{50} of 1.4 and 24.9 mM, respectively (Iwai *et al.*, 2006). In a separate *in vitro* study chlorogenic acid inhibited maltase and sucrase activity with IC_{50} of 2.9 and 2.2 mM, respectively in a non-competitive manner (Ishikawa *et al.*, 2007). Chlorogenic acid has also showed α-amylase inhibition activity, with IC_{50} values of 0.07-0.08 mM (Narita and Inouye, 2009). Proanthocyanidins from a persimmon peel extract showed α-amylase and α-

glucosidase inhibition activity. Proanthocyanidins polymers proved higher inhibition on α-amylase activity, 100 μg/ml polymers or oligomers inhibited activity by 54% vs 5%, respectively. Oligomers showed a stronger inhibition on α-glucosidase activity, 25 μg of oligomers or polymers per millilitre inhibited activity by 90% vs 30%, respectively. Data suggest that the degree of polymerisation might play important role on the inhibition activity on α-amylase (Lee *et al.*, 2007), previous studies have suggested proanthocyanidins act by binding to the protein rather than the active site (Grussu *et al.*, 2011). In a separate study proanthocyanidins from a maritime pine bark extract showed α-glucosidase inhibition activity (IC_{50}= 5 μg/ml), the inhibitory effect of the extract was stronger in fractions containing proanthocyanidins with higher degree of polymerisation, when oligomers and dimer and trimers where compared. In the same study the flavan-3-ol catechin showed inhibition of α-glucosidase activity in a weaker manner (IC_{50}= 52 μg/ml) (Schafer and Hogger, 2007).

1.6.5.2 Glucose uptake by the intestine: *in vitro* studies

In addition to potential polyphenol-induced delayed digestive breakdown of carbohydrates, inhibition of glucose transporters (SGLT1 and GLUT2) by apple extracts and phlorizin (Manzano and Williamson, 2010, Schulze *et al.*, 2014) as well as quercetin glycosides (Ader *et al.*, 2001) have been showed in *in vitro* systems.

A flavonoid- and phenolic acid-rich apple extract showed 50% inhibition of total glucose uptake (1 mM) when 300 mg of apple solid extract were tested in Caco-2 cells. The apple extract also inhibited the GLUT-mediated glucose uptake by 50% at 146 mg of apple extract; results suggest a greater effect on the GLUT-mediated glucose uptake component in this *in vitro* system (Manzano and Williamson, 2010). However the effective doses represent supra-physiological concentrations, which are unlikely to be present in gut lumen under normal dietary conditions unless pureed and concentrated. Individual flavonoids and phenolics acid have also been tested in *in vitro* systems. The flavan-3-ols catechin and epicatechin (100 μM) when tested in the Caco-2 cells system caused a significant decrease in GLUT-mediated glucose uptake (1 mM) (Johnston *et al.*, 2005), although they did not inhibit total glucose uptake (SGLT1- and GLUT-mediated) either at 100 μM, nor at 50 μM as tested in a separate study (Shimizu, 2000). Results suggest that the flavan-3-ols monomers exerted inhibition of GLUT transporters in the Caco-2 model. The flavonol quercetin 100 μM inhibited GLUT-mediated glucose uptake (1 mM) by 75% but no total glucose uptake in Caco-2 cells (Johnston *et al.*,

2005) and quercetin-3-O-rhamnoside inhibited total glucose uptake (1 mM) and apical to basolateral glucose transport with IC_{50} values of 380 and 31 μM, respectively (Manzano and Williamson, 2010).

The dihydrochalcones phlorizin and phloretin have been proven to inhibit total and GLUT-mediated glucose uptake, respectively in Caco-2 cells *in vitro* model. Phlorizin 100 μM inhibited total glucose uptake by 50% (Johnston *et al.*, 2005) while the same degree of inhibition was showed on total glucose uptake by phloretin 468 μM (Manzano and Williamson, 2010), and in a separate study phloretin 100 μM inhibited by 47% GLUT-mediated glucose uptake (1 mM) but not total glucose uptake (Johnston *et al.*, 2005). Results suggest a higher inhibitory effect on total glucose uptake by phlorizin and higher inhibitory effect on GLUT-mediated glucose uptake by phloretin, which has been well documented (Wright *et al.*, 2011). In the same *in vitro* model, 50% inhibition of total glucose uptake was exerted by chlorogenic acid 1.3 mM (Manzano and Williamson, 2010), and similar results were shown in a separate study using everted rat gut sacs, with 48 % inhibition of total glucose uptake by chlorogenic acid 1 mM (Ishikawa *et al.*, 2007). On rat small intestine bush border membrane vesicles chlorogenic acid 1 mM inhibited total glucose uptake by 80 % (Welsch *et al.*, 1989).

In an *in vitro* model of oocytes injected to express SGLT1, 0.24 μg/ml of a phlorizin-rich apple extract showed 50% inhibition of glucose uptake (Schulze *et al.*, 2014). Phlorizin is a well-known competitive inhibitor of SGLT1 with a Ki ~200 nM (Wright *et al.*, 2011) and its aglycone phloretin has also showed a SGLT1 inhibitory effect in a non-competitive manner (Hirayama *et al.*, 2001, Schulze, 2014). Other flavonoids contained in apple and apple derived food, as flavonols quercetin (IC_{50}= 0.62 mM), quercetin-3-O-galatoside (IC_{50}= 1.32 mM) and quercetin-4'-O-glucoside (IC_{50}= 0.17 mM) have showed inhibition of SGLT1 (Kottra and Daniel, 2007, Schulze *et al.*, 2015). In the same *in vitro* system but with oocytes injected to express GLUT2, quercetin inhibited sugar uptake with an IC_{50} value of 13 μM (Kwon *et al.*, 2007).

1.7 Proanthocyanidins

Proanthocyanidins (also known as condensed tannins) is a complex group of the flavanol subclass, consisted of monomers, dimers, oligomers and polymers of catechin and/or epicatechin units (**Figure 1.4**) that may also contain gallate esters. Proanthocyanidins consisting exclusively of catechin/epicatechin units are named procyanidins and are the most common type in plants. Fruit and vegetables are among the main food sources of proanthocyanidins, but nut skin contains the highest quantities (Crozier *et al.*, 2012).

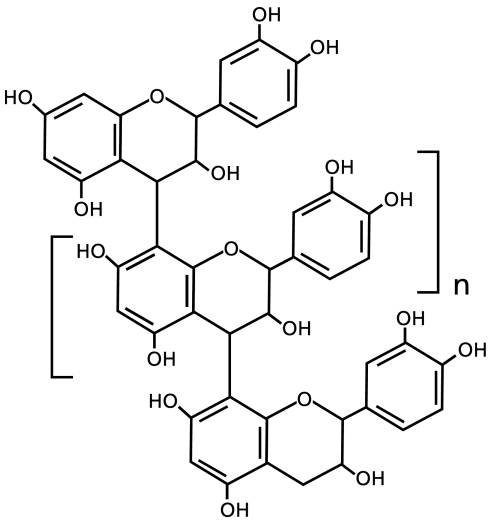


Figure 1.4 General structure of proanthocyanidin oligomer

As mention in the previous section, proanthocyanidin intakes have been associated with reduced risk of T2D and *in vitro* studies have showed inhibition of digestive enzymes activity. Bioavailability studies on proanthocyanidin have showed a decreased permeability into intestinal cells and high interaction with the plasma membrane. Probable factors affecting the permeability are molecular weight, polymerisation degree, hydrophilic characteristic and affinity for luminal proteins (Deprez *et al.*, 2001). The permeability of proanthocyanidins tends to decrease when polymerisation increase, proanthocyanidins dimers and oligomers (2-5 mers) have demonstrated being absorbable but proanthocyanidins with a higher polymerisation degree (>5 mers) has showed nil permeability into intestinal cells in studies with animals and humans (Neilson *et al.*, 2016). High molecular phenolic molecules as proanthocyanidins, with a size up to 10 kDa (Yanagida *et al.*, 2003), present a significant number of hydroxyl groups in their structure what give them hydrophilic characteristics. It is known that

hydrophilic flavonoids may interact by hydrogen bonding with the polar head groups of the phospholipids in the outer leaflet of the plasma membrane (Oteiza *et al.*, 2005), the degree of interaction appears to be mediated by the presence of hydroxyl groups in the flavonoid compound. A research group has suggested that big molecules of polyphenols may interact with phospholipids coating the surface and covering proteins inserted in the plasma membrane (Tarahovsky, 2008). Their capacity to bind to membrane phospholipids and a possible interaction with proteins by covalent and non-covalent bonds (Kroll *et al.*, 2005) might explain some of their effects. Although consumption of proanthocyanidin-rich food (such as tea and wine) has showed to inhibits the absorption of non-heme dietary iron through the gut, due to their affinity for iron (III) and the non-specific binding of proanthocyanidin to phospholipids and proteins possibly interfere with other nutrients transporters, its nutritional implication is probably of limited consequences if proanthocyanidins are consumed in adequate amounts (Scalbert *et al.*, 2000).

1.8 Overall aims

Polyphenol-rich fruit intake is associated with reduced risk of T2D. Epidemiological studies suggest that berry and apple intakes in particular correlate with beneficial health effects. UK dietary guidelines recommend that the average intake of fruit and vegetables is increased from the current estimates of 4.1 portions per day (adults aged 19-64 y (Bates *et al.*, 2014)) to "5-a-day", but there is no special recommendation for polyphenol-rich fruit and/or vegetables. Therefore, the **first aim** of this project was to investigate whether adherence to UK dietary guidelines and increasing the consumption of fruit and vegetables to at least 5 portions/day led to an actual increased intake in polyphenols associated with beneficial effects on health **(Chapter 2)**.

Fruit polyphenols may help to delay glucose absorption and thereby control glucose levels following a carbohydrate-containing meal or beverage. Many of the clinical trials testing effects of polyphenol-rich fruits on postprandial glucose have used the entire fruit, fruit purées, fruit juices or similar foodstuff that include in the food matrix other components as fibre that might interfere with glucose response, besides some trials using carbohydrate loads that might not simulate normal dietary habits. The **second aim** of this project was to determine whether plasma glucose concentrations were reduced by consumption of highly purified blackcurrant and apple extracts immediately before a high-carbohydrate (starch and sucrose) test meal **(Chapter 3)**.

Blackcurrant and apple–based test meals have been shown to lower postprandial glycaemia *in vivo*, and some reports suggest that polyphenols derived from these fruits can inhibit glucose uptake in *in vitro* models of the small intestine. However concentrations tested *in vitro* frequently exceed achievable concentrations in normal diets therefore the **third and final aim** of this thesis was to identify the mechanisms of glucose uptake inhibition by physiological concentrations of blackcurrant and apple extracts, consumed in our clinical trials, using the intestinal sugar transport model Caco-2/TC-7 cells and the *Xenopus laevis* oocytes system **(Chapter 4)**.

The specific aims and hypotheses are detailed within the individual chapters, but the overarching thesis is that regular dietary intakes of blackcurrant and apple polyphenols may help reduce risk of type 2 diabetes and cardiovascular disease by reducing the rate at which glucose is absorbed thereby lowering blood glucose concentrations postprandially.

Chapter 2

Dietary polyphenol intakes in a healthy, middle-aged UK population: comparison of a dietary pattern following UK government guidelines with a traditional UK dietary pattern using a food frequency questionnaire, a four-day food diary and 24 h urinary biomarkers.

2.1 Introduction

The World Health Organization recommends consumption of 5 portions of fruit and vegetables per day (WHO, 1990), UK dietary guidance follows this recommendation (NHS, 2005) but the National Diet and Nutrition Survey (NDNS) (Bates *et al.*, 2014) reveals that consumption of fruit and vegetables are below the recommendation in 70 % of UK adult population. UK government guidelines also recommend an increase from 23 g to 30 g for the average adult population intake of dietary fibre (SACN, 2015) and although there is not a specific guideline on whole grains, data from the NDNS revealed that UK adult population intake is below other countries recommendations (Mann *et al.*, 2015). High intake of fruit and vegetables, and dietary fibre/whole grains are associated with a reduced risk of chronic diseases (Boeing *et al.*, 2012, Muraki *et al.*, 2013, Mursu *et al.*, 2014, Buil-Cosiales *et al.*, 2016, Aune *et al.*, 2016); the increased consumption of different components present in these food groups, like fibre (Ötles and Ozgoz, 2014), micronutrients (Woodside *et al.*, 2005) and polyphenols (Rodriguez-Mateos *et al.*, 2014), have been identified as responsible for some of the beneficial effects.

Dietary intakes of polyphenols are derived from a wide range of foods: fruits, vegetables, cereals and plant-based beverages such as tea, coffee, red wine, fruit juices and cocoa represent the main dietary sources (Manach *et al.*, 2004). Epidemiological studies have shown a negative relationship between ingestion of polyphenols and cardiovascular disease (Hooper *et al.*, 2008, Jennings *et al.*, 2012, McCullough *et al.*, 2012, Cassidy *et al.*, 2013, van Dam *et al.*, 2013), cancer (Neuhouser, 2004, Arts and Hollman, 2005, Fink *et al.*, 2007, Yuan, 2011) and type 2 diabetes (Wedick *et al.*, 2012, Curtis *et al.*, 2012, van Dam *et al.*, 2013, Jennings *et al.*, 2014, Jacques *et al.*, 2013). Although epidemiological studies rely on the estimation of polyphenol intakes this is a difficult enterprise, since the most common dietary assessment methods employed for estimation, such as FFQ, 24-HR and food diaries, depend on the ability of the subject to accurately describe their own food intake and the availability of adequate databases for the content of polyphenols on food (Zamora-Ros *et al.*, 2014). At present, the most common databases employed to assess polyphenol intakes are the USDA (Nutrient Data Laboratory, 2004, Bhagwat, 2008, Bhagwat, 2013) and Phenol-Explorer (Neveu *et al.*, 2010, Rothwell *et al.*, 2012, Rothwell *et al.*, 2013) which provide information on the content of 35 flavonoids in 506 food items and 502 polyphenols (of the 4 classes) in 459 food items, respectively.

New techniques for intake estimation have been examined; these include innovative technologies for measuring dietary intakes in epidemiological studies, like personal digital assistant and camera and tape recorder (Illner *et al.*, 2012), as well as biomarker approaches, like urine and plasma metabolites. Urinary excretion of polyphenols has proved to be a valid and reliable biomarker for the intake of polyphenols (Mennen *et al.*, 2008, Perez-Jimenez *et al.*, 2010a), fruit and vegetables (Nielsen *et al.*, 2002, Krogholm *et al.*, 2004), polyphenol-rich beverages (Ito *et al.*, 2005, Mullen *et al.*, 2010) and polyphenol-rich food (Mennen *et al.*, 2006, Edmands *et al.*, 2015). The presence of polyphenol metabolites in urine is closely related to the quantity consumed and the overall metabolism in the body, however different polyphenols may produce common metabolites therefore the biomarker selected must reflects the specific intake of the parent polyphenol in question (Spencer *et al.*, 2008).

No dietary recommendations exist for polyphenols, although the advantages and disadvantages of this approach have been debated (Williamson and Holst, 2008, Lupton *et al.*, 2014). It is assumed that a dietary pattern consistent with current dietary guidelines will be richer in polyphenols than the average UK dietary pattern due to increased intakes of fruit and vegetables and whole grains. However, not all fruits and vegetables are polyphenol rich (Perez-Jimenez *et al.*, 2010b), and in fact the majority of UK average polyphenol intakes are determined by tea, coffee, and chocolate intakes (Zamora-Ros *et al.*, 2015).

2.2 Hypothesis

Intakes of total polyphenols and subclasses of polyphenols will be greater in a free-living population following UK dietary guidelines when compared to average traditional UK diet.

2.3 Methodology

The Cardiovascular risk REduction Study: Supported by an Integrated Dietary Approach (CRESSIDA) was a parallel-designed, randomised, controlled trial funded by the Food Standards Agency/Department of Health (UK) (N02047), sponsored by King's College London and approved by the St. Thomas' Hospital Research Ethics Committee (10/H0802/24) (Reidlinger *et al.*, 2015). Recruitment commenced in 2010 and the last participant completed the trial in July 2012. This trial was registered at Current Controlled Trial (http://www.controlled-trials.com/ISRCTN) as ISRCTN92382106. The primary aim of the study was to compare a traditional UK diet with what is currently believed to be a cardioprotective diet (following UK government dietary guidelines), a dietary guidelines (DG) diet, to assess effects on well-established and emerging CVD risk factors. Changes in blood pressure and plasma total cholesterol/HDL cholesterol ratio were primary outcomes as these are powerful and well-established predictors of future CVD risk. Secondary outcomes included endothelial function, arterial stiffness, C-reactive protein and measures of insulin sensitivity.

Participants between the age of 40-70 y were chosen because risk increases markedly with age and an upper age limit of 70 y was selected because absolute annual risk of CVD exceeds 2 % beyond that age in the majority of people and a higher proportion are receiving medication on daily basis. In selecting the control diet, it was decided to compare the DG diet adhering to current UK dietary guidelines with a nutritionally balanced traditional UK control diet that would be acceptable to study participants and formulated with familiar foods (full cream milk, cheese, butter, meat and meat products, non-wholegrain cereals) which reflected typical UK intakes of fruit and vegetables (3 portions/d), had a high saturated fatty acids content (14 % energy), unrestricted intakes of salt and sugar and low intakes of oily fish.

The lead researcher was Prof Thomas Sanders, co-investigators were Dr Wendy Hall, Dr Sarah Berry, Prof Phil Chowienczyk and Dr Paul Seed, and study investigators included Dr Dianne Reidlinger, Dr Julia Darzi, Ms Virginia Govoni, Dr Louise Goff,

Mr Robert Gray and Ms Laura O'Sullivan. The author's participation consisted in the adaptation of the dietary database provided by Dr Dianne Reidlinger for its *post hoc* use in the analysis of flavonoids and total polyphenols consumption.

2.3.1 Participants

A total of 165 healthy men and women aged 40–70 years were recruited, and 162 completed the intervention (64 M, 97 F) (**Figure 2.1**).

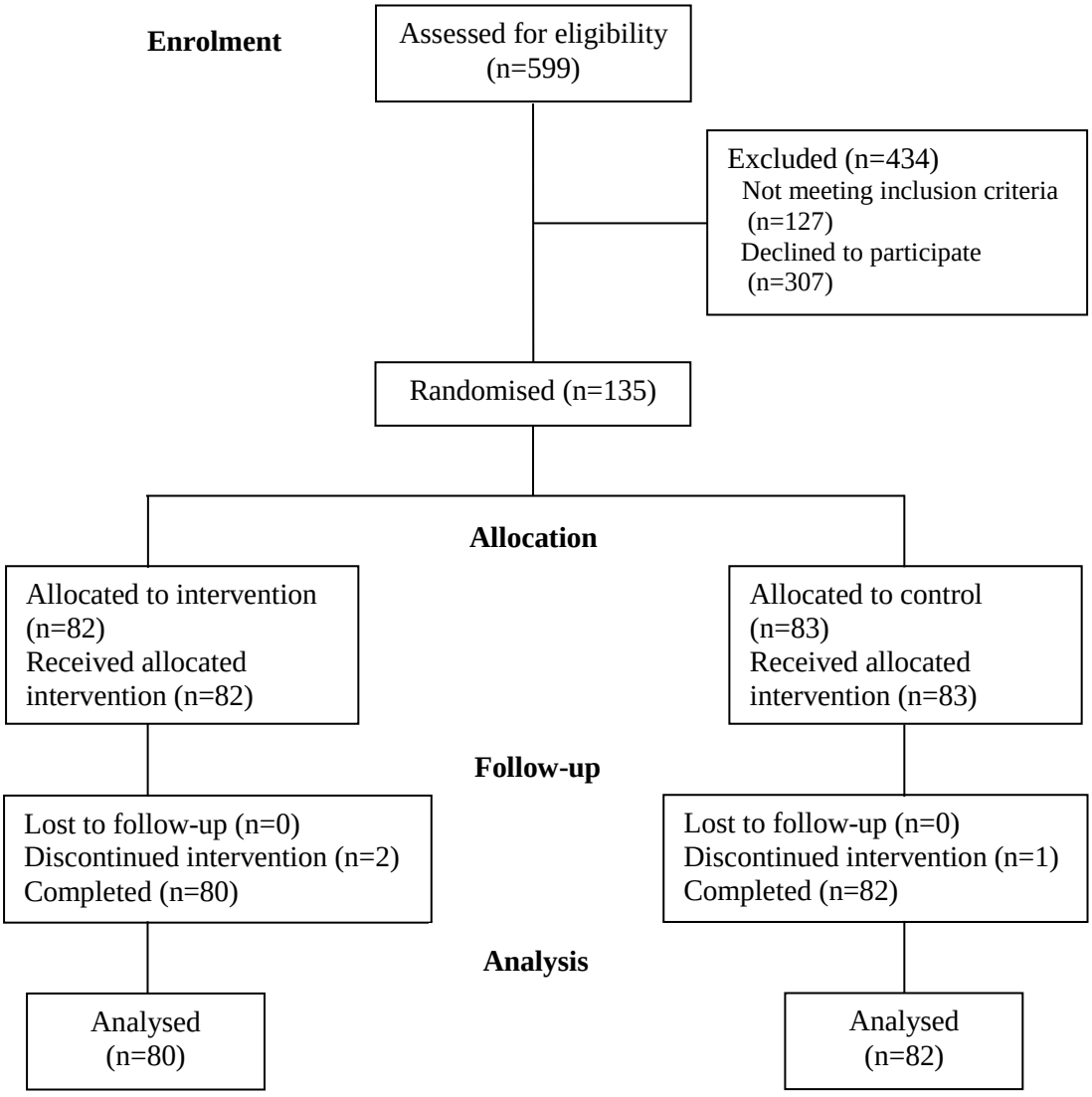


Figure 2.1 Flow of participants through the CRESSIDA study

Participants received dietary advice in person at baseline and at week 4, and by phone at week 6 and 8. Dietary assessment included two food frequency questionnaires (FFQ), two four-day food diaries (4-DFD) and two 24-HR recalls (**Figure 2.2**).

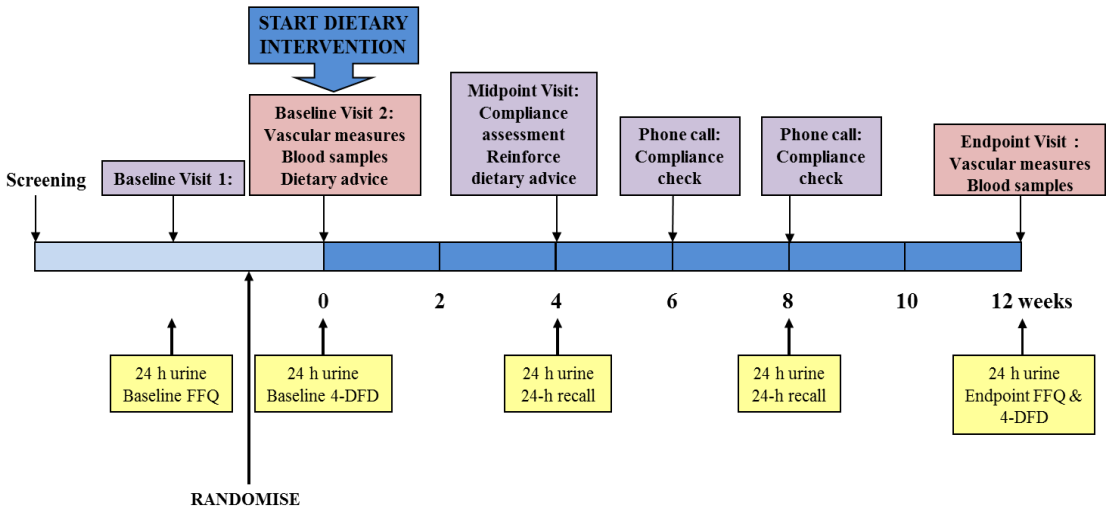


Figure 2.2 CRESSIDA study timeline

2.3.2 Dietary advice

Participants randomised to the DG diet group were given advice to consume 2 portions of fish per week (1 of which should be oily); to consume reduced fat dairy products instead of full-fat products; to replace fats rich in saturated fatty acids with spreads/oils low in saturated fatty acids and high in monounsaturated fatty acids; to increase intake of fruit and vegetables to 5 portions/day; to restrict salt intake to <6 g/d (<100 mmol/d); to reduce intake of free[1] sugars; and to increase whole grains intake to >50% of cereal intake. On the other hand, the participants randomised to the control diet (reflecting the average traditional British diet) were advised to restrict intake of oily fish to less than 1 per month; to consume oils, spreads and dairy products that were higher in saturated fatty acids; to restrict intake of fruit and vegetables to 3 portions/day; there was no restriction on salt or free sugars intake; and to consume refined cereals. The study included provision of spread, oil, whole grain pasta, rice and cereal bars, high fibre breakfast cereal (oats, muesli, etc.), almonds and macadamia nuts and tinned oily fish to the intervention group and spread, oil and refined cereals as pantry items to the control group (Reidlinger, 2015).

[1] CRESSIDA study was done before SACN 2015 where definition of 'free sugars' was stablished instead of the previous 'non-milk extrinsic sugars' term and guidelines on sugar and fibre were changed.

2.3.3 Food frequency questionnaires (FFQ)

Dietary intake of flavonoids was quantified from 322 FFQs (n=161) (EPIC-Norfolk FFQ v.6) (Bingham *et al.*, 1994) at baseline and endpoint of the 12-wk intervention; data from one participant had to be omitted because both FFQ were not completed. Flavonoid intake was estimated from 6 subclasses and most representative compounds for each: flavanones (hesperetin, naringenin, eriodictyol), anthocyanins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin), flavones (apigenin, luteolin), flavonols (quercetin, kaempferol, myricetin, isorhamnetin), flavan-3-ols (catechin, gallocatechin, epicatechin, epigallocatechin, epicatechin-3-gallate, epigallocatechin-3-gallate) and polymers (theaflavins, thearubigins and proanthocyanidins). In total 32 types of flavonoids were analysed using a database created and provided by Prof Aedin Cassidy at University of East Anglia (Jennings *et al.*, 2012) and modified for the requirements of the FFQ´s analysis, so that the flavonoid content of 130 food items were analysed.

FFQ registers the frequency of consumption of specific foods, of standard portion size, in a month. There were nine answer options which varied from "never or less than once in a month" to "six or more per day". To calculate the total intake of subclasses of flavonoids, frequencies were converted to a daily portion (**Table 2.1**) and then multiplied for the flavonoid content in each specific food item; the results were summed for each subclass and for each participant.

Table 2.1 Example of conversion of FFQ answers to a daily portion on the item strawberries, raspberries and kiwi fruit

Subject	**Food item** Strawberries, raspberries, kiwi fruit		**Portion per day** Strawberries, raspberries, kiwi fruit	**Daily portion** (g/d) (100 g in database-portion x portion per day)[1]
003	1	Never or less than once a month	0	0
015	3	Once a week	0.14	14
040	2	1-3 per month	0.06	6
140	4	2-4 per week	0.43	43
028	6	Once a day	1	100
225	5	5-6 per week	0.785	78.5

[1]Values for different foodstuffs in the same food variable were averaged out to create a single food item.

For calculation of total intake of flavonoids per subject, Microsoft Excel 2007 for Windows was used, different equations were used to import data from database, multiply flavonoid content in each specific food per daily portion and finally to sum up values on each subclass and individual flavonoid for each participant (**Table 2.2**).

Additional food not included in the FFQ standard items but mentioned by participants as food they eat more than once a week were also included in the calculation of flavonoid intakes.

For assessment of the main food sources of the subclasses and individual flavonoids pivot tables were created using Microsoft Excel 2007 for Windows. Final calculations on flavonoid intakes were included in three separate tables; one for baseline estimations in control and intervention group, one for endpoint estimations on control group and one more for endpoint estimations on intervention group (**Figure 2.3**).

Table 2.2 Example of excel spreadsheet used to calculate flavonoid intakes on the item strawberries, raspberries and kiwi fruit

Subject	Food item	g/d	Total flavonoids (mg)	Flavanones (mg)	Anthocyanidins (mg)	Flavanols (mg)	Flavonols (mg)	Flavones (mg)	Polymers (mg)
003	Strawberries, raspberries, kiwi fruit	0	0	0	0	0	0	0	0
015	Strawberries, raspberries, kiwi fruit	14	11.53	0.012	3.34	0.53	0.13	0.052	7.47
040	Strawberries, raspberries, kiwi fruit	6	4.95	0.005	1.43	0.23	0.06	0.022	3.20
140	Strawberries, raspberries, kiwi fruit	43	35.46	0.037	10.26	1.63	0.41	0.159	22.96
028	Strawberries, raspberries, kiwi fruit	100	82.46	0.086	23.86	3.80	0.96	0.370	53.38
225	Strawberries, raspberries, kiwi fruit	78.5	64.73	0.067	18.73	2.98	0.75	0.290	41.91

Microsoft Excel - Pivot tables main sources

Row Labels	Sum of Total flavonoids	Sum of Flavanones	Sum of Anthocyanins	Sum of flavanols	Sum of flavonols	Sum of flavones	Sum of Polymers
Tea (cup)	48.86%	0.00%	0.00%	76.57%	26.01%	0.00%	52.29%
Apples	12.22%	0.00%	6.45%	5.83%	8.31%	4.11%	15.28%
Peanuts or other nuts	5.15%	0.00%	5.26%	0.00%	0.00%	0.00%	7.10%
Oranges, satsumas, mandarins	5.06%	84.81%	0.00%	0.00%	1.22%	28.33%	0.00%
Grapes	4.79%	0.00%	25.75%	1.34%	1.40%	6.84%	4.90%
Strawberries, raspberries, kiwi fruit	4.61%	0.08%	31.68%	1.47%	0.94%	4.50%	4.30%
Peaches, plums, apricots	3.69%	0.00%	3.84%	2.17%	1.29%	0.00%	4.53%
Wine (glass)	2.72%	2.17%	8.71%	3.04%	2.24%	10.32%	2.32%

Figure 2.3 Example of pivot table for assessment of main food sources of flavonoids in CRESSIDA population based on FFQ analysis

2.3.4 Four-day food diary (4-DFD)

Dietary intake of polyphenols was quantified from 322 x 4-DFD (n=161), at baseline and endpoint of the 12-wk intervention. A polyphenol food composition database was created using Phenol-Explorer (Neveu *et al.*, 2010, Rothwell *et al.*, 2013, Rothwell *et al.*, 2012) and USDA databases (Bhagwat, 2013, Bhagwat, 2008, Nutrient Data Laboratory, 2004), and extended using polyphenol retention factors and recipes provided by participants. If a recipe was not provided by participant, a standard recipe was obtained from either the UK food tables (Finglas, 2015), a UK food industry recipe book (Campbell, 2012), the BBC Good Food website (Worldwide, 2009) or additional websites specialised in UK recipes and other countries typical cuisine.

Polyphenol intake was estimated for four classes of polyphenols: flavonoids, lignans, phenolic acids and stilbenes. An additional group of polyphenols were included under the name "other polyphenols"; since Phenol-Explorer (PE) grouped here a series of compounds as alkylmethoxyphenols, alkylphenols, curcuminoids, furanocoumarins, tyrosols among others.

The flavonoid intake was analysed in eight subclasses: anthocyanins, dihydrochalcones, flavanols (flavanol-3-ols monomers and theaflavins), proanthocyanidins, flavanones, flavones, flavonols and isoflavones, for each subclass a range of two to eleven of the most representative individual compounds were selected. For lignans subclass four individual compounds were analysed: pinoresinol, lariciresinol, secoisolariciresinol and matairesinol. For phenolic acids subclass two groups were analysed: hydroxybenzoic acids and hydroxycinnamic acids, for each group a range of four to six of the most representative individual compounds were selected. For stilbenes subclass one individual compound was selected and analysed: resveratrol. A total of 52 individual polyphenols were analysed and 1141 food items were included in the final database (**Table 2.3**). The selection of the database (USDA/PE) was based on availability and polyphenol profile description of the food item, if both databases described the food the one with the more detailed profile was used.

The food diary registers the grams or millilitres of food ingested by participant per day, to calculate the total intake of polyphenols subclasses and individual compounds, grams or millilitres of food were multiplied for the polyphenols content in each specific food item; the results were summed for each individual compound and subclasses for each participant.

The creation of the database for analysis of total polyphenol intake was a meticulous process, involving the description of polyphenol contents on raw food as fruit and vegetables than can be consumed without exposure to any cooking (boil, fry, bake, roast, etc.) or physical process (peeling, freezing, storing, etc.). Retention factors, when available, were used to modify the polyphenol content on particular food after experiencing cook or physical modifications, retention factors indicate how much of the content of raw food is keep after the alteration (for examples see **Table 2.4**). The process of creation started on November 2012 and the database was finished on April 2015.

In addition to the values of individual food items, 403 recipes were broken down in order to estimate the total polyphenol content on them (**Table 2.5**). As previously mentioned recipes were obtained from different sources; in total 118 recipes were obtained from participants, 93 from UK food tables, 20 from a UK food industry recipe book, 78 from the BBC Good Food website and 94 from other websites.

For calculation of total intake of polyphenols per subject, Microsoft Excel 2007 for Windows was used, different functions were used to import data from databases (food polyphenol content and food dietary intake); multiply polyphenol content in each specific food per grams or millilitres ingested and finally to sum up values on each subclass and individual polyphenols for each participant (**Table 2.3**). Although nutrient intakes on the CRESSIDA population were calculated using Weighed Intake analysis Software Package (Tinuviel Software, WISP version 3.0), the software allowed only the storage of information for 30 polyphenols to be quantified limiting the analysis planned and leading to the preference for Excel, allowing unlimited number of polyphenols to include in the final polyphenol intake analysis.

For assessment of the main food sources of the subclasses and individual polyphenols pivot tables were created using Microsoft Excel 2007 for Windows. Final calculations on polyphenols intakes were included in three separate tables; one for baseline estimations in control and DG group, one for endpoint estimations on control group and one more for endpoint estimations on intervention group (**Figure 2.4**).

Table 2.3 Small section of the database created to calculate the total polyphenol intake estimated by 4-DFD

Food code	Food name	Database	Food weight (g or ml)	Flavonoids (mg)	Lignans (mg)	Phenolic acids (mg)	Stilbenes (mg)	Total polyphenols (mg)
13148	Alfalfa, sprouts, raw	USDA	100	1.70	0.00	0.00	0.00	1.70
8293	Almond cake, homemade, recipe	RFP	495[1]	194.78	0.00	11.99	0.00	206.77
10214	American muffins, chocolate	M&W	532[1]	3744.37	0.00	51.92	0.17	3796.46
11279	Apple pie, pastry top and bottom	BBC	1926.5[1]	1294.20	0.00	262.69	0.00	1556.89
30376	Apple sponge	OW	584[1]	129.42	0.00	39.17	0.00	168.59
14012	Apples, eating, average, raw	PE	100	146.11	0.00	19.89	0.00	166.00
14014	Apples, eating, average, raw, peeled	PE	100	129.42	0.00	19.91	0.00	149.33
11	Apricot, jam	PE	100	0.89	0.00	0.00	0.00	0.89
14025	Apricot, raw	PE	100	26.18	0.00	5.33	0.00	31.51

USDA, United States Department of Agriculture databases; RFP, recipes from participants; M&W, recipes from UK food tables; BBC, BBC Good food website, OW; other websites, PE; Phenol-Explorer database.

[1] Grams per recipe

Table 2.4 Example of retention factor used to modify polyphenol contents

Food code	Food name	Database	g	RF	Flavonoids (mg)	Flavonols (mg)	Quercetin (mg)	Phenolic acids (mg)	Caffeic acid (mg)	Total polyphenols (mg)
13342	Shallots, raw	PE	100		112.00	112.00	112.00	1.00	1.00	113.00
43	Shallots, fried	PE	100	0.79	88.48	88.48	88.48	1.00	1.00	89.48
13446	Carrots, old, raw	PE	100		0.72	0.45	0.00	19.51	18.83	20.23
47	Carrots, old, fried	PE	100	1.22	0.72	0.45	0.00	23.66	22.97	24.38
13009	Old potatoes, average, raw	PE	100		0.06	0.06	0.005	28.35	28.35	28.41
13014	Old potatoes, average, boiled	PE	100	0.82	0.06	0.06	0.005	23.25	23.25	23.31

RF, retention factor; PE, Phenol-Explorer database

Table 2.5 Example of breakdown of recipes and quantification of polyphenol content

Food code	Food name	g in recipe	Flavonoids	Proanthocyanidins	Flavanones	Flavones	Flavonols	Other polyphenols	Phenolic acids	Total polyphenols
8174	**Shepherd's Pie (RFP)**									
18040	Beef, mince, extra lean, raw	500	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0
13461	Tomatoes, canned, whole contents	400	1.74	0.00	1.26	0.00	0.48	0.00	13.18	14.92
13822	Curry powder	9	6.67	6.67	0.00	0.00	0.00	25.67	0.00	32.34
43	Shallots, fried	60	53.09	0.00	0.00	0.00	53.09	0.00	0.60	53.69
13451	Celery, fries	80	3.61	0.00	0.00	3.12	0.49	0.00	0.00	3.61
13466	Leeks, fried	100	2.98	0.00	0.00	0.00	2.98	0.00	0.00	2.98
47	Carrots, old, fried	85	0.61	0.00	0.00	0.23	0.38	0.00	20.11	20.72
13014	Old potatoes, average, boiled	800	0.48	0.00	0.00	0.00	0.48	0.00	186.00	186.48
17516	Tomato puree	15	0.63	0.00	0.00	0.00	0.63	0.00	0.00	0.63
12002	Skimmed milk, pasteurised	150	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0
5017	Study spread 80% fat	20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0
	Total	**2219**	**69.81**	**6.67**	**1.26**	**3.35**	**58.53**	**25.67**	**219.89**	**315.37**

RFP, recipes from participants

Microsoft Excel - Pivot table endpoint-intervention (food gruop & g)

Row Labels	Sum of Flavonoids, total	Sum of Lignans, total	Sum of Other polyphenols, total	Sum of Phenolic acids, total	Sum of Stilbenes, total	Sum of Polyphenols, total
Tea, black, infusion, average	44.25%	1.52%	0.00%	19.55%	0.00%	35.19%
Coffee, infusion, average	0.00%	0.00%	7.53%	47.10%	0.00%	14.84%
Chocolate, plain	6.46%	0.00%	0.00%	0.10%	0.40%	4.28%
Apples, eating, average, raw	5.41%	0.00%	0.00%	1.56%	0.00%	4.04%
Red wine (average)	4.17%	0.00%	0.00%	0.00%	0.00%	2.74%
Tea, green, infusion	3.56%	0.15%	0.00%	1.22%	0.00%	2.72%
Strawberries, raw	2.26%	0.00%	0.00%	0.42%	4.65%	1.62%

Figure 2.4 Example of pivot table for assessment of main food sources of polyphenols in CRESSIDA population based on 4-DFD analysis

2.3.5 Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

A previously published protocol for polyphenol analysis in urine (Ito *et al.*, 2005) was adapted and developed by the author for the quantification of ten aglycone metabolites of polyphenols in 24 h urine samples from the CRESSIDA study participants.

A subsample of the CRESSIDA study was selected for the analysis; the selection was based in the intake of fruit and vegetables (F&V) reported in the endpoint FFQ, 45 samples from participants in the control group reporting the lowest intakes of F&V, and 46 samples from participants in the intervention group reporting the highest intakes of F&V were analysed. Quantification of endpoint samples allowed the comparison between groups at the end of the dietary intervention; the aim was to detect greater amounts of polyphenols excreted in urine by participants randomised to the intervention group, in order to determine whether this mirrored the dietary intake pattern observed in FFQ and 4-DFD analysis.

One or two of the most representative aglycones of different subclasses of polyphenol were selected for their quantification, the ten phenolic compounds analysed were as follows; phloretin (dihydrochalcones), epicatechin (flavan-3-ols), hesperetin and eriodictyol (flavanones), luteolin (flavones), quercetin (flavonols), daidzein (isoflavones), gallic and vanillic acid (phenolic acids) and enterolactone as a product of microbial metabolism of lignans in colon. The phenolic compounds selected for quantification had proved to be convenient biomarkers of polyphenol intake (Perez-Jimenez *et al.*, 2010b) with a high recovery and good correlation with fruit and vegetable intakes when estimated by FFQ, 24-h recalls or food diaries (Edmands *et al.*, 2015). The major food sources of the ten phenolics compounds are fruit, vegetables and wholegrain products, or represent the main metabolites of parent glucosides present in the previously mentioned foods (**Table 2.6**).

Table 2.6 Main food sources of the ten phenolic compounds selected for LC-MS/MS analysis

Polyphenol	**Main food sources**[*]	**Main food sources in UK adult population**[§]
Phloretin	Apple	Apple
Epicatechin	Cocoa, dark and milk chocolate, apple, green tea, black tea, blackberry	Black tea, apples and pears, milk beverages, cakes and sweet pies, chocolate candy/bars (Knaze *et al.*, 2012)
Hesperetin	Orange, blood and blond, lemon, tangerine, peppermint	Fruit juices, citrus fruit, carbonated/soft drinks (Zamora-Ros *et al.*, 2015)
Eriodictyol	Peppermint, oregano, lemon, sour orange, almond	Main sources of flavanones: citrus fruits, juices, wine (Zamora-Ros *et al.*, 2011a)
Luteolin	Oregano, thyme, sage, artichoke, cabbage, brussels, black olive, olive oil, pistachio	Main sources of flavones: herbal tea, juices, condiments, citrus fruits, wine, olives (Zamora-Ros *et al.*, 2011a)
Quercetin	Chokeberry, black tea, red raspberry, greencurrant, dark chocolate, red lettuce, red onion, broccoli	Main sources of flavonols: black tea, apples and pears, onion, garlic, soups (Zamora-Ros *et al.*, 2011a)
Daidzein	Soy tempeh, soy tofu, soybean, soy milk, black bean, soy flour	Main source of isoflavones: bread[2] and bread rolls, breakfast cereals[2], vegetable dishes[2], soy milk (Mulligan *et al.*, 2013)
Gallic acid	Cloves, green and red chicory, black tea, blackberry, red wine	Black tea, wine, mixed fruits (Zamora-Ros *et al.*, 2015)
Vanillic acid	Dried sweet basil, dried dates, dried thyme, cranberry, green olive	Main sources of phenolic acids: coffee, black tea, breakfast cereals, herbal tea, wine, apples and pears, stone fruits (Zamora-Ros *et al.*, 2015)

Table 2.6 continued

Polyphenol	Main food sources*	Main food sources in UK adult population§
Enterolactone[1]	Main source of lignans: linseeds, sesame seed, sunflower seed, dried date, cashew nut, peanut	Main source of lignans: Seeds, vegetable oils, condiments, bread (Zamora-Ros *et al.*, 2015)

*****Data obtained from Phenol-Explorer and USDA databases
§ Data obtained from the European Prospective Investigation into Cancer and Nutrition (EPIC) study
[1] Enterolactone is a metabolite of lignans
[2] Contain soya/textured vegetable protein/tofu/tempeh foods

2.3.5.1 Materials

Purified standards and reagents used in the processing of 24 h urine samples are listed in **Table 2.7**. Stock solutions were prepared for all the purified phenolic compounds, two milligrams of each of the following compounds; phloretin, epicatechin, eriodictyol, hesperetin, luteolin, quercetin, gallic acid, vanillic acid and enterolactone were diluted in one millilitre of methanol, two milligrams of daidzein in one millilitre of dimethyl sulfoxide and one milligram of Catechin-2, 3, 4-$^{13}C_3$ (internal standard) in one millilitre of methanol. Stock solutions were kept at -40 ^{o} C or -80 ^{o} C (internal standard). Enzyme β-glucoronidase/sulfatase (0.05 g) type H-5 from *Helix Pomatia* was diluted in one millilitre of 0.2% sodium chloride solution to have a working solution of ~50,000 units per millilitre; the enzyme solution was stored at -40 ^{o} C.

Table 2.7 List of material used to process 24 h urine samples

Material	**Supplier details**
Phloretin	Santa Cruz Biotechnology, cat. no. sc-3548
Epicatechin	Santa Cruz Biotechnology, cat. no. sc-205672
Eriodictyol	Sigma-Aldrich, cat. no. 74565-5MG-F
Hesperetin	Santa Cruz Biotechnology, cat. no. sc-252878
Luteolin	Santa Cruz Biotechnology, cat. no. sc-203119A
Quercetin	Sigma-Aldrich, cat. no. Q4951-10G
Daidzein	Santa Cruz Biotechnology, cat. no. sc-24001
Gallic acid	Santa Cruz Biotechnology, cat. no. sc-205704
Vanillic acid	Santa Cruz Biotechnology, cat. no. sc-251422
Enterolactone	Sigma-Aldrich, cat. no. 45199-5MG-F
(+/-) Catechin-2, 3, 4-$^{13}C_3$	Sigma-Aldrich, cat. no. 719579-1MG
β-glucoronidase type H-5 from *Helix Pomatia*	Sigma-Aldrich, cat. no. G1512-100KU
Methanol	Fisher Scientific CAS-No 67-56-1
Dimethyl sulfoxide (DMSO)	Fisher Scientific CAS-No 67-68-5
HPLC-grade water	Fisher Scientific CAS-No 7732-18-5
Ethyl acetate	Fisher Scientific CAS-No 141-78-6

2.3.5.2 Working solutions

Working solutions were freshly prepared the same day that samples were processed; mixed polyphenols working solution was prepared by taking 10 µl of each stock solution (ten purified compounds), in total 120 µl and made up to 10 ml with HPLC-grade water for a final concentration of 2 ng/µl. The internal standard working solution were prepared by taking 12 µl of stock solution and made up to 4 ml with HPL-grade water for a final concentration of 3 ng/µl. Working solutions were used to prepare calibration curves.

2.3.5.3 Urine collection

Urine samples were collected at five time-points (**Figure 2.2**), but LC-MS/MS analysis was only performed on endpoint samples, collected at week 12 of the intervention to coincide with dietary assessment by 4-DFD. A urine collection protocol was developed based on standardised methods used in previous studies (Sadler *et al.*, 2012). Participants were asked to collect all urine from the second pass of the day until the first pass of the following day in 2.5 L bottles containing boric acid as preservative, as used in the EPIC study for preservation of urine stored at -20 ^{o} C for at least 15 years prior to polyphenol analysis (Achaintre *et al.*, 2016). Participants were asked to mark the meniscus of the urine on each bottle and then carefully transfer 10 ml aliquot from each bottle using a 10 ml Urine Monovette (Sarstedt, cat no 10.252), labelled with the relevant bottle number. The remaining urine was disposed of and the Urine Monovette(s) containing the urine aliquot(s) and empty urine bottle(s) marked with the urine meniscus were conserved. The total urine volume was recorded and 7 x 1.5 ml aliquots of pooled 24 h urine samples were stored at -80^{o}C until analysis. Completeness of urine collection was measured using recovery of para-aminobenzoic acid according to a standard protocol (Bingham and Cummings, 1983), participants were asked to take 3 x 80 g para-aminobenzoic acid at three regularly spaced intervals throughout the day of urine collection.

2.3.5.4 Enzymatic hydrolysis

Hydrolysis of metabolites in urine started with the acidification of 250 µl of urine sample with 20 µl of acetic acid (0.58 M), following by addition of 1300 units of β-glucoronidase/sulfatase contained in 26 µl of 0.2% sodium chloride solution, for hydrolysis of glucuronide and sulphated metabolites. Catechin-$^{13}C_3$ (3 ng/µL) was used

as internal standard, 100 µl were added to each sample. The mixture was incubated in a shaking water bath at 37 ^{o} C, 120 rpm for 1.5 h, after incubation the mixture was acidified by addition of 10 µl of hydrochloric acid (HCl, 1 M) followed by a liquid-liquid extraction. Extraction was performed twice by adding 400 µl and 300 µl of ethyl acetate, mixed by vortex for 20 min and centrifuged at 3000 rpm, 25 ^{o} C for 10 min. The two organic layers were pooled and evaporated until dryness under N_2 followed by reconstitution in 100 µl of 40 % methanol immediately before injection. Batches of urine samples were hydrolysed, dried under N_2, and kept at -80 ^{o} C for 1-4 days until their reconstitution with 40% methanol previous to injection.

2.3.5.5 LC-MS/MS quantification

Analysis of urine samples was performed on a HPLC system Hewlett-Packard series 1100 binary pump, coupled to a triple quadrupole mass spectrometer, Micromass Quattro LC (Micromass, Limited), operating in negative electrospray ionisation (ES-) mode, N_2 gas flow of 550 L/h, cone voltage 35 V and desolvation temperature 350 ^{o} C. The analysis was performed in multiple reaction monitoring (MRM) mode. Ionisation and fragmentation was optimised for each polyphenol by direct infusion of a standard solution, 200 ng/ml of pure polyphenol in 5 mM of ammonium acetate in 45% aqueous methanol solution. Specific values for collision energy parameters were identified for each polyphenol. Parent (precursor) and daughter (product) ions and retention time are shown in **Figure 2.5**. Peak for each polyphenol identity was established by both the characteristic parent and daughter ion pair and retention time. The chromatographic column for separation was a Zorbax SB-C18 (2.1 x 50mm, 3.5mm, Agilent) maintained at 25 ^{o} C. Solvent A consisted of water with 0.1% v/v of formic acid and solvent B acetonitrile with 0.1 % v/v of formic acid were run in a 95/5% proportion at a flow rate of 0.2 ml/min, injection volume of 10 µl and a run time of 15 min. The chromatographic conditions of elution were as follows: 0–1 min, 5 % solvent B; 1–4 min, increase solvent B from 5% to 10%; 4–5 min, increase solvent B from 10% to 90%; 5-5.2 min, decrease solvent B from 90% to 5%, 5.2-15 isocratic for 9.8 min. Peak areas of the 10 polyphenols were plotted against the internal standard response. A good linearity (r^2 0.970–0.990) was observed for almost all the polyphenols quantified except for luteolin, r^2= 0.922. All samples were run in the same batch and all chromatograms were processed automatically by MassLynx Mass Spectometry Software (MASSLYNX^{TM} version 3.5) using the same processing integrate parameters, peak-to-peak amplitude and peak detection. Manual integration was performed only when peaks were difficult

to differentiate after the automatic integration. Values for intra-assay coefficients of variability varied from, 5–21% for the different compounds; phloretin 7.5%, epicatechin 6%, hesperetin 21.9%, eriodictyol 9.6%, luteolin 9.8%, quercetin 10.8%, daidzein 3.1%, gallic acid 21.4%, vanillic acid 14.4% and enterolactone 5.1%. Final concentrations of phenolics compounds were estimated after adjustment by urinary volume and corrected by milligram of creatinine.

2.3.5.6 Calibration curves

Calibration curves were prepared by spiking HPLC-grade water with 2.5, 5, 12.5, 25, 50, 100, 150, 200 and 250 µl of mixed polyphenols working solution and 100 µl of internal standard working solution, the calibration curve range was 5-500 ng/ml. Spiked HPLC-grade water samples were treated with enzyme and extracted with ethyl acetate as were the urine samples, spiked samples were injected in duplicate at each concentration level. Limits of quantification were established using the spiked samples for calibrations curves; minimum detected value were 5 ng/ml for phloretin, epicatechin, eriodictyol, hesperetin, luteolin, daidzein, gallic acid and vanillic acid and 10 ng/ml for quercetin and enterolactone.

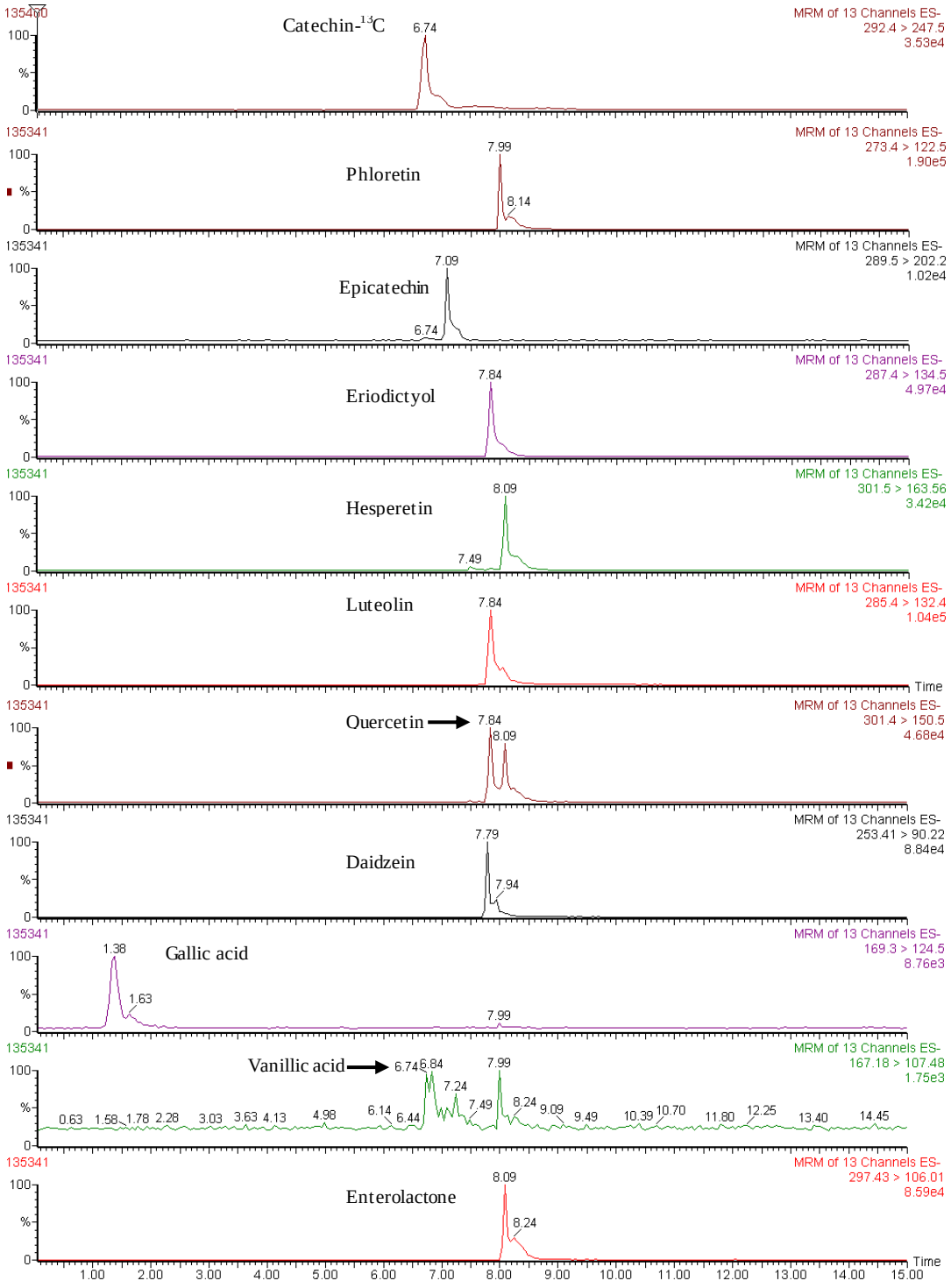


Figure 2.5 Parent ions, daughter ions and retention times of polyphenols standards

2.3.6 Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) v.21. Normality of data distribution was evaluated visually by inspection of histograms and normal Q-Q plots and verified using the Kolmogorov-Smirnov test. In normally distributed data independent sample t-tests were conducted to compare groups at both baseline and endpoint. If there was a statistically significant difference at baseline, one-way analysis of variance with baseline value as covariate (ANCOVA) was conducted to find differences between groups at endpoint. Comparison between groups at endpoint was done to determine the differences in polyphenol intakes in populations adhering to the different dietary interventions rather than investigating changes in polyphenol intakes over time. For non-normally distributed data, Mann-Whitney U test was conducted to find differences between groups at both baseline and endpoint. If there was a statistically significant difference at baseline, Mann-Whitney U tests were conducted on the difference (endpoint – baseline) between groups.

2.4 Results

2.4.1 Dietary flavonoid intakes estimated by food frequency questionnaire

Descriptive data of the CRESSIDA study population at baseline is shown in **Table 2.8**, the largest contributor to the total flavonoid intake in this population is represented by polymeric flavonoids (theaflavins and proanthocyanidins) accounting for 70% of the total intake following by the flavan-3-ols class with a 14% the rest 16% was accounted for the subclasses flavanones, anthocyanidins, flavones and flavonols.

Table 2.8 Characteristics of the CRESSIDA study population (n=161)

Characteristic	**Baseline**
Gender (F/M)	97/64
Age[1] (years)	53 ± 8
BMI[1] (kg/m^2)	26.1 ± 3.9
Total flavonoids[1] (mg/d)	664 ± 343
Flavanones[2] (mg/d)	23 (34)
Anthocyanidins[2] (mg/d)	19 (26)
Flavones[2] (mg/d)	2 (2)
Flavonols[2] (mg/d)	35 (24)
Flavan-3-ols[2] (mg/d)	90 (103)
Polymeric flavonoids[2] (mg/d)	231 ± 171

[1] Values are mean ± SD
[2] Values are median (interquartile range)

There were no differences between groups for any of the flavonoid dietary intakes (total and subclasses) at baseline. Dietary intake of total flavonoids and the subclasses anthocyanidins, flavones, flavan-3-ols and polymeric flavonoids were statistically significantly higher in the group following the DG diet at endpoint (**Table 2.9**); flavanones and flavonols did not differ between intervention groups at endpoint.

Table 2.9 Daily intake of flavonoids, as estimated by FFQ (n=161)

Flavonoids (mg/d)	**Control (n=81)**					**Dietary Guidelines (n=80)**				
Subclasses	Baseline		Endpoint		Change	Baseline		Endpoint		Change
Total flavonoids[1]	618	(337)	621	(347)	3 (-61, 67)	711	(344)	**752**[4]	(299)	41 (-20, 102)
Flavanones[2]	23	(7, 43)	25	(10, 43)	0 (-11, 11)	22	(11, 39)	27	(8, 49)	2 (-7, 20)
Anthocyanidins[3]	16	(3)	15	(2)	1 (1, 1)	20	(3)	**31**[4]	(2)	2 (1, 2)
Flavones[2]	2	(2, 4)	3	(2, 4)	0 (-1, 1)	3	(2, 3)	**3**[5]	(2, 4)	1 (0, 2)
Flavonols[2]	35	(22, 47)	39	(21, 54)	4 (-3, 9)	36	(24, 47)	39	(31, 50)	4 (-3, 11)
Flavan-3-ols[2]	84	(38, 142)	85	(35, 146)	1 (-7, 12)	93	(54, 143)	**97**[5]	(80, 143)	0 (-12, 30)
Polymeric[1]	437	(249)	441	(261)	4 (-46, 53)	510	(265)	**523**[4]	(220)	13 (-33, 61)

[1] Values are mean (SD); Change mean (95% CI)
[2] Values are median (interquartile range)
[3] Values are geometric mean (geometric standard deviation); Change, mean (95% CI)
[4] Independent sample t-tests were carried out to find differences between groups at baseline and endpoint, there were no differences between groups at baseline. There were statistically significant differences between groups at endpoint, $P<0.05$
[5] Mann-Whitney U tests were carried out to find differences between groups at baseline and endpoint, there were no differences between groups at baseline. There were statistically significant differences between groups at endpoint, $P<0.05$

There were no differences between groups for any of the individual compounds of the flavanones subclass at baseline and endpoint (**Table 2.10**).

Table 2.10 Daily intake of flavanones, as estimated by FFQ (n=161)

Flavonoids (mg/d)		**Control (n=81)**			**Dietary Guidelines (n=80)**		
Subclass	Individual	Baseline	Endpoint	Change	Baseline	Endpoint	Change
Flavanones[1]		23 (7, 43)	25 (10, 43)	0 (-11, 11)	22 (11, 39)	27 (8, 49)	2 (-7, 20)
	Hesperetin[1]	12 (3, 24)	14 (6, 24)	1 (-6, 8)	13 (7, 22)	14 (5, 28)	0 (-5, 10)
	Naringenin[1]	9 (2, 16)	9 (3, 16)	0 (-4, 4)	9 (4, 16)	12 (3, 21)	1 (-4, 9)
	Eriodictyol[1]	0.04 (0.01, 0.07)	0.04 (0.01, 0.07)	0 (-0.01, 0.03)	0.04 (0.01, 0.09)	0.01 (0.01, 0.06)	-0.01 (-0.05, 0.00)

[1] Values are median (interquartile range)
There were no differences between groups at baseline and endpoint, by Mann-Whitney U test, $P<0.05$

In the anthocyanidins subclass there was a difference between groups in the intake of petunidin at baseline, with higher intake in the group randomised to the DG diet, there were no differences between groups for any other of the individual compounds at baseline. There were statistically significant differences between groups in all the individual compounds of anthocyanidins at endpoint, higher intakes were observed in the group following the DG diet (**Table 2.11**). For petunidin intake data on changes (endpoint-baseline) for both groups were analysed by Mann-Whitney U test due to the initial difference in the intervention group and the fact that a non-normal distribution prevented analysis by ANCOVA with baseline as covariate. The main food sources of anthocyanidins in the DG group at endpoint, within the limitations of the FFQ categories available, were as follows: strawberries, raspberries, kiwi fruit (32%), grapes (26%) and wine (9%). As for the individual compounds the main food sources follow the same tendency; pelargonidin and cyanidin with strawberries, raspberries, kiwi fruit counted for 92% and 37% respectively, and delphinidin, malvidin, peonidin and petunidin with grapes as main food source and counted for 42%, 70%, 60% and 33% respectively.

Table 2.11 Daily intake of anthocyanins, as estimated by FFQ (n=161)

Flavonoids (mg/d)		**Control (n=81)**			**Dietary Guidelines (n=80)**		
Subclass	Individuals	Baseline	Endpoint	Change	Baseline	Endpoint	Change
Anthocyanidins[1]		16 (3)	15 (2)	1 (1, 1)	20 (3)	**31**[3] (2)	2 (1, 2)
	Cyanidin[1]	6 (3)	7 (3)	1 (1, 1)	8 (3)	**13**[3] (2)	2 (1, 2)
	Delphinidin[2]	1 (0, 2)	1 (0, 1)	0 (0, 0)	1 (1, 2)	**2**[4] (1, 2)	0 (0, 1)
	Malvidin[2]	4 (2, 9)	4 (2, 9)	0 (-2, 1)	5 (3, 11)	**8**[4] (1, 15)	1 (-1, 5)
	Pelargonidin[2]	2 (1, 5)	2 (1, 5)	0 (-3, 1)	2 (1, 5)	**5**[4] (1, 9)	0 (-1, 4)
	Peonidin[2]	0.4 (0.2, 0.9)	0.4 (0.2, 0.9)	-0.1 (-0.2, 0.2)	0.6 (0.3, 1)	**0.7**[4] (0.4, 1)	0.1 (-0.1, 0.4)
	Petunidin[2]	0.7 (0.4, 1)	0.6 (0.4, 1)	0.1 (-0.3, 0.3)	0.9[4] (0.5, 1)	1 (1, 2)	**0.1 (-0.1, 0.5)**[5]

[1] Values are geometric mean (geometric standard deviation); Change mean (95% CI).
[2] Values are median (interquartile range)
[3] Independent sample t-tests were carried out to find differences between groups at baseline and endpoint, there were no differences between groups at baseline. There were statistically significant differences between groups at endpoint, $P<0.05$
[4] Mann-Whitney U tests were carried out to find differences between groups at baseline and endpoint, there were differences between groups at baseline on petunidin intakes. There were statistically significant differences between groups at endpoint, $P<0.05$
[5] Mann-Whitney U test was carried out on change values to compare groups at endpoint given the statistical significant difference between groups at baseline. There was a statistically significant difference between groups at endpoint on petunidin intakes, $P<0.05$

There were no differences between groups for any of the individual compounds of the flavones subclass at baseline. There was a statistically significant difference between groups in luteolin intake at endpoint, an increased intake was observed in the group following the DG diet (**Table 2.12**). The main food sources of flavones in the DG group at endpoint were ranked as follows: oranges, satsumas, mandarins (28%), sweet peppers (19%), green salad, lettuce, cucumber, celery (13%), wine (10%) and grapes (7%). Luteolin main food sources in the DG group at endpoint ranked the same way as for flavones subclass except for grapes and wine were grapes contribute with a higher percentage than wine.

Table 2.12 Daily intake of flavones, as estimated by FFQ (n=161)

Flavonoids (mg/d)		**Control (n=81)**					**Dietary Guidelines (n=80)**				
Subclass	Individuals	Baseline		Endpoint		Change	Baseline		Endpoint		Change
Flavones[1]		2.3	(1.5, 3.5)	2.7	(1.5, 3.7)	0.1 (-0.6, 0.8)	2.5	(1.7, 3.4)	**3.2**[2]	(2.0, 4.4)	0.6 (-0.4, 1.5)
	Apigenin[1]	0.5	(0.3, 0.8)	0.6	(0.3, 0.9)	0.0 (-0.1, 0.2)	0.6	(0.3, 1.0)	0.6	(0.4, 0.9)	0.0 (-0.2, 0.2)
	Luteolin[1]	1.6	(0.9, 2.8)	2.0	(1.0, 2.8)	0.2 (-0.6, 0.6)	1.8	(1.0, 2.7)	**2.3**[2]	(1.4, 3.4)	0.5 (-0.3, 1.5)

[1] Values are median (interquartile range)
[2] Mann-Whitney U tests were carried out to find differences between groups at baseline and endpoint, there were no differences between groups at baseline. There were statistically significant differences between groups at endpoint, $P<0.05$

There were no differences between groups for any of the individual compound of the flavonols subclass at baseline. There was a statistically significant difference between groups in myricetin intake at endpoint; an increase was observed in the group following the DG diet (**Table 2.13**). The major dietary sources of flavonols in the DG group at endpoint were as follows: onions (35%), tea (26%) and apples (8%). Myricetin main food sources were; tea (64%), wine (13%), tomatoes (5%).

Table 2.13 Daily intake of flavonols, as measure by FFQ (n=161)

Flavonoids (mg/d)	**Control (n=81)**			**Dietary Guidelines (n=80)**		
Subclass Individuals	Baseline	Endpoint	Change	Baseline	Endpoint	Change
Flavonols[1]	35 (22, 47)	39 (21, 54)	4 (-3, 9)	36 (24, 47)	39 (31, 50)	4 (-3, 11)
Quercetin[1]	26 (17, 37)	29 (17, 43)	4 (-2, 8)	27 (20, 37)	31 (25, 42)	3 (-2, 9)
Kaempferol[1]	4 (2, 8)	5 (2, 8)	0 (-1, 1)	6 (3, 8)	5 (4, 8)	0 (-1, 2)
Myricetin[1]	1.6 (0.8, 2.6)	1.6 (0.8, 2.7)	0.1 (-0.2, 0.3)	2.0 (1.3, 2.6)	**1.9**[2] (1.6, 2.6)	0.1 (-0.3, 0.6)
Isorhamnetin[1]	0.1 (0.04, 0.20)	0.10 (0.04, 0.21)	0.01 (-0.02, 0.07)	0.10 (0.05, 0.22)	0.1 (0.04. 0.25)	0 (-0.03, 0.06)

[1] Values are median (interquartile range)

[2] Mann-Whitney U tests were carried out to find differences between groups at baseline and endpoint, there were no differences between groups at baseline. There were statistically significant differences between groups at endpoint, $P<0.05$

In the flavan-3-ols subclass there were no differences between groups for any of the individual compounds at baseline. There were statistically significant differences between groups in the (+)-catechin, (-)-epicathechin-3-gallate and (-)-epigallocatechin-3-gallate intakes at endpoint, an increased was observed in the group following the DG diet (**Table 2.14**). The major dietary sources of flavan-3-ols in the DG group at endpoint were as follows: tea (77%), apples (6%) and bananas (4%). The main food sources for (+)-catechin in the DG group at endpoint were bananas (29%), tea (27%) and wine (13%), for (-)-epicathechin-3-gallate were; tea (99%), grapes (0.4%) and peaches, plums, apricots (0.4%) and for (-)-epigallocatechin-3-gallate; tea (98%), apples (1%) and strawberries, raspberries, kiwi fruit (0.4%).

Table 2.14 Daily intake of flavan-3-ols, as estimated by FFQ (n=161)

Flavonoids (mg/d)		**Control (n=81)**			**Dietary Guidelines (n=80)**		
Subclass	Individuals	Baseline	Endpoint	Change	Baseline	Endpoint	Change
Flavan-3-ols[1]		84 (38, 142)	85 (35, 146)	1 (-7, 12)	93 (54, 143)	**97**[2] (80, 143)	0 (-12, 30)
	(+)-Catechin[1]	13 (9, 18)	13 (9, 18)	0 (-3, 3)	14 (11, 18)	**15**[2] (12, 21)	1 (-2, 5)
	(+)-Gallocatechin[1]	3 (1, 5)	3 (1, 5)	0 (0,0)	3 (1, 5)	3 (3, 5)	0 (0,0)
	(-)-Epicatechin[1]	14 (9, 18)	14 (9, 20)	0 (-2, 3)	16 (12, 23)	15 (12, 21)	0 (-4, 4)
	(-)-Epigallocatechin[1]	21 (7, 36)	21 (6, 37)	0 (-2, 2)	21 (10, 37)	22 (20, 37)	0 (-2, 3)
	(-)-Epicatechin-3-O-gallate[1]	15 (3, 27)	15 (3, 27)	0 (0, 0)	15 (6, 27)	**15**[2] (15, 27)	0 (0, 2)
	(-)-Epigallocatechin-3-O-gallate[1]	23 (5, 42)	23 (4, 42)	0 (0, 1)	23 (9, 42)	**24**[2] (23, 42)	0 (0, 4)

[1] Values are median (interquartile range)
[2] Mann-Whitney U tests were carried out to find differences between groups at baseline and endpoint, there were no differences between groups at baseline. There were statistically significant differences between groups at endpoint, $P<0.05$

There were no differences between groups for any of the individual compounds of polymeric flavonoids at baseline. There were statistically significant differences between groups in the proanthocyanidins (PA) and proanthocyanidins polymers intake at endpoint, an increased was observed in the group following the DG diet (**Table 2.15**). The major dietary sources of polymeric flavonoids in the DG group at endpoint were as follows: tea (52%), apples (15%) and peanuts or other nuts (7%). The main food sources for PA were apples (27%), peanuts or other nuts (13%), tea (13%) and grapes (8%); for PA polymers were apples (29%), grapes (21%), peanuts or other nuts (14%) and strawberries, raspberries, kiwi fruit (12%).

Table 2.15 Daily intake of polymeric flavonoids, as estimated by FFQ (n=161)

Flavonoids (mg/d)	**Control (n=81)**					**Dietary Guidelines (n=80)**				
Subclass Individuals	Baseline		Endpoint		Change	Baseline		Endpoint		Change
Polymeric[1]	436.7	(249.0)	440.6	(260.9)	3.9 (-45.6, 53.3)	509.8	(264.9)	**523.1**[4]	(220.0)	13.3 (-34.0, 60.6)
Theaflavin[2]	3.9	(0.7, 7.1)	3.9	(0.7, 7.1)	0.0 (0.0, 0.1)	3.9	(1.6, 7.1)	3.9	(3.9, 7.1)	0.0 (0.0, 0.6)
Theaflavin 3-O-gallate[2]	3.1	(0.5, 5.6)	3.1	(0.5, 5.6)	0.0 (0.0, 0.1)	3.1	(0.5, 5.6)	3.1	(3.1, 5.6)	0.0 (0.0, 0.5)
Theaflavin 3'-O-gallate[2]	3.8	(0.7, 6.8)	3.8	(0.7, 6.8)	0.0 (0.0, 0.1)	3.8	(1.5, 6.8)	3.8	(3.8, 6.8)	0.0 (0.0, 0.6)
Theaflavin 3,3'-O-digallate[2]	4.4	(0.8, 7.9)	4.4	(0.8, 7.9)	0.0 (0.0, 0.1)	4.4	(1.8, 7.9)	4.4	(4.4, 7.9)	0.0 (0.0 ,0.6)
Thearubigins[2]	203.3	(34.9, 365.9)	203.3	(34.9, 365.9)	0.0 (0.0, 3.3)	203.3	(81.3, 365.9)	203.3	(203.3, 365.9)	0.0 (0.0, 30.1)
Proanthocyanidins (PA)[3]	238.5	(1.9)	250.8	(1.7)	1.1 (0.9, 1.2)	282.3	(1.8)	**296.0**[4]	(1.6)	1.0 (0.9, 1.2)
PA monomers[2]	50.2	(30.4, 69.4)	48.8	(31.4, 71.6)	2.9 (-10.6, 10.4)	57.7	(36.9, 71.8)	49.7	(34.7, 65.2)	-4.5 (-17.1, 8.1)
PA dimers[3]	38.0	(1.8)	39.4	(1.7)	2.2 (1.9, 2.5)	44.3	(1.8)	41.8	(1.5)	2.0 (1.7, 2.2)
PA trimers[3]	18.1	(2.0)	19.7	(1.7)	0.4 (0.3, 0.4)	21.2	(1.9)	19.8	(1.7)	0.3 (0.3, 0.4)
PA 4-6 mers[3]	50.3	(2.0)	53.6	(1.8)	1.9 (1.6, 2.1)	57.9	(2.0)	58.3	(1.7)	1.7 (1.5, 2.0)
PA 7-10 mers[3]	28.6	(2.0)	30.3	(1.8)	0.6 (0.5, 0.7)	33.7	(2.0)	35.5	(1.8)	0.6 (0.5, 0.7)
PA polymers (>10 mers)[3]	52.2	(2.1)	55.0	(1.8)	1.3 (1.1, 1.5)	62.8	(2.2)	**85.2**[4]	(1.9)	1.6 (1.4, 1.9)

[1]Values are mean (SD)
[2]Values are median (interquartile range); Change mean (95% CI)
[3]Values are geometric mean (geometric standard deviation)
[4]Independent sample t-tests were carried out to find differences between groups at baseline and endpoint, there were no differences between groups at baseline. There were statistically significant differences between groups at endpoint, $P<0.05$

2.4.2 Dietary polyphenol intakes estimated by 4-day food diary (4-DFD)

Descriptive data of the CRESSIDA study population at baseline is shown in **Table 2.16**, the largest contributor to the total polyphenol intake in this population is represented by the flavonoids subclass counting for a 67% of the total intake followed by the phenolic acids with a 21% the rest 12% was accounted for by lignans and stilbenes and other polyphenols. The major dietary sources of polyphenols for the CRESSIDA study participants estimated at baseline were: black tea (35%), coffee (16%), fruit and vegetables (15%) and plain chocolate (4%).

Table 2.16 Characteristics of the CRESSIDA study population (n=161)

Characteristic	Baseline
Gender (F/M)	97/64
Age[1] (years)	53 ± 8
BMI[1] (kg/m^2)	26.1 ± 3.9
Total polyphenols[2] (mg/d)	1183 (868)
Flavanoids[2] (mg/d)	790 (648)
Lignans[2] (mg/d)	0.4 (3.9)
Phenolic acids[2] (mg/d)	243 (29)
Stilbenes[2] (mg/d)	0.08 (0.63)
Other polyphenols[2] (mg/d)	19 (29)

[1] Values are mean ± SD
[2] Values are median (IQR)

There were no differences between groups for the subclasses: lignans, phenolics acids, stilbenes and other polyphenols at baseline. There were statistically significant differences between groups in the total polyphenols and flavonoids intakes at baseline, with higher values in the group randomised to the DG diet. There were statistically significant differences between groups in lignans, phenolic acids and other polyphenols intakes at endpoint, with higher intakes in the group following the DG diet (**Table 2.17**). The main food sources of lignans in the DG group at endpoint were as follows: linseeds (46%), hummus (16%) and sesame oil (14%); for phenolic acids: coffee (47%), black tea (22%), herbal tea (3%) and apples (2%); and for other polyphenols: muesli, luxury fruit and nuts (41%), wholemeal spaghetti (23%) and coffee (6%).

Table 2.17 Daily intake of polyphenols, as estimated by 4-DFD (n=161)

Polyphenols (mg/d))	**Control (n=82)**					**Dietary Guidelines (n=79)**				
Subclasses	Baseline		Endpoint		Change	Baseline		Endpoint		Change
Total polyphenols	1009	(634, 1468)	977	(621, 1597)	17 (-216, 223)	1273[1]	(868, 1644)	1290	(775, 1894)	83 (-390, 252)
Flavonoids	645	(337, 988)	675	(409, 1035)	27 (-128, 182)	919[1]	(590, 1143)	788	(536, 1314)	0.9 (-262, 218)
Lignans	0.34	(0.18, 2.45)	0.26	(0.13, 0.44)	-0.05 (-0.42, 0.07)	0.52	(0.21, 4.43)	**0.42[1]**	(0.20, 5)	0.03 (-0.37. 2)
Phenolic acids	228	(121, 502)	217	(114, 467)	3 (-83. 53)	248	(164, 576)	**300[1]**	(155, 554)	3 (-52, 71)
Stilbenes	0.05	(0.01, 0.54)	0.09	(0.02, 0.85)	0 (-0.07, 0.16)	0.11	(0.02, 0.73)	0.19	(0.05, 1)	0.03 (-0.10, 0.34)
Other polyphenols	21	(10, 49)	14	(6, 24)	-6 (-23, 2)	16	(8, 29)	**34[1]**	(16, 50)	11 (-6, 33)

All values are median (interquartile range)
[1]Mann-Whitney U tests were carried out to find differences between groups at baseline and endpoint, there were no differences between groups at baseline. There were statistically significant differences between groups at endpoint, $P<0.05$

There was a statistically significant difference between groups in proanthocyanidins intake at baseline, with greater intake in the group randomised to the DG diet. There were no differences between groups for any other flavonoids subclass at baseline. There was a statistically significant difference between groups in isoflavones intake at endpoint with higher intakes in the group following the DG diet (**Table 2.18**). The main food sources of isoflavones in the group following the DG diet at endpoint were as follows: soy drinks/fat free/fruit (65%), soy yogurt/fruit (16%) and plain soy milk (14%).

Table 2.18 Daily intake of flavonoids, as estimated by 4-DFD (n=161)

Polyphenols (mg/d)		**Control (n=82)**					**Dietary Guidelines (n=79)**				
Subclass	Individuals	Baseline		Endpoint		Change	Baseline		Endpoint		Change
Flavonoids		645	(337, 988)	675	(409, 1035)	27 (-128, 182)	919[1]	(590, 1143)	788	(536, 1314)	0.9 (-262, 218)
	Anthocyanidins	46	(7, 125)	29	(13, 108)	-2 (-46, 32)	49	(16, 129)	69	(19, 122)	1 (-50, 57)
	Dihydrochalcones	1	(0, 3)	2	(0, 4)	0 (-1, 2)	2	(0, 3)	2	(1, 5)	0 (-2, 2)
	Flavanols	272	(89, 488)	261	(99, 468)	1 (-48, 79)	367	(116, 530)	340	(135, 540)	-8 (-86, 69)
	Proanthocyanidins	160	(77, 266)	187	(99, 271)	12 (-42, 98)	247[1]	(138, 337)	214	(127, 305)	10 (-108, 113)
	Flavanones	34	(5, 61)	24	(2, 51)	-1 (-22, 12)	23	(3, 58)	26	(7, 77)	3 (-14, 20)
	Flavones	5	(3, 10)	5	(1, 12)	0 (-3, 4)	5	(2, 11)	4	(2, 10)	0 (-3, 3)
	Flavonols	62	(40, 103)	68	(42, 100)	5 (-16, 21)	78	(45, 105)	75	(52, 117)	4 (-15, 24)
	Isoflavones	0.26	(0.10, 6)	0.13	(0.06, 0.39)	-0.11 (-5, 0.06)	0.25	(0.13, 16.04)	**0.52[1]**	(0.06, 51)	0.05 (-0.21, 41)

All values are median (interquartile range)

[1]Mann-Whitney U tests were carried out to find differences between groups at baseline and endpoint, there were differences between groups at baseline for flavonoids and proanthocyanidins intakes. There was a statistically significant difference between groups at endpoint, on isoflavone intakes, $P<0.05$

There were no differences between groups for any of the individual compounds in the anthocyanidins subclass at baseline. There were statistically significant differences between groups in cyanidin, malvidin and peonidin intakes at endpoint, with higher intakes in the group following the DG diet (**Table 2.19**). In the DG group at endpoint, the major food sources of cyanidin are as follows: raspberries (46%), blackcurrant juice/concentrated (10%) and plums (8%); for malvidin: red wine (73%), grapes (20%) and blueberries (6%) and for peonidin: red wine (52%), grapes (21%), plums (11%) and blueberries (8%). These results confirm the findings from FFQ analysis where berries (strawberries, raspberries and grapes) are ranked as the major food sources of the anthocyanidins individual compounds.

Table 2.19 Daily intake of anthocyanidins, as estimated by 4-DFD (n=161)

Polyphenols (mg/d)		**Control (n=82)**			**Dietary Guidelines (n=79)**		
Subclass	Individuals	Baseline	Endpoint	Change	Baseline	Endpoint	Change
Anthocyanidins		46 (7, 125)	29 (13, 108)	-2 (-46, 32)	49 (16, 129)	69 (19, 122)	1 (-50, 57)
	Cyanidin	4 (1, 33)	4 (1, 20)	0 (-7, 10)	8 (2, 21)	**7**[1] (2, 33)	0 (-13, 20)
	Malvidin	5 (0, 37)	2 (0, 39)	0 (-15, 3)	9 (0, 53)	**11**[1] (0, 45)	0 (-13, 8)
	Peonidin	1 (0, 3)	1 (0, 3)	0 (-2, 0)	2 (0, 4)	**2**[1] (0, 4)	0 (-1, 1)
	Pelargonidin	2 (0, 5)	2 (0, 8)	0 (-3, 4)	1 (0, 11)	3 (0, 16)	0 (-1, 9)
	Delphinidin	4 (1, 13)	3 (1, 11)	0 (-4, 6)	6 (1, 13)	4 (1, 12)	0 (-6, 6)
	Petunidin	1 (0, 4)	0 (0, 4)	0 (-2, 0)	1 (0, 6)	1 (0, 6)	0 (-2, 1)

All values are median (interquartile range)
Mann-Whitney U tests were carried out to find differences between groups at baseline and endpoint, there were no differences between groups at baseline.
[1]There were statistically significant differences between groups at endpoint, $P<0.05$

There was a statistically significant difference between groups in phloretin intakes at baseline, with higher intake in the group randomised to the DG diet. There were no differences between groups for phlorizin and dihydrochalcones at baseline. There were no differences between groups for any of the individual compounds of the dihydrochalcones subclass at endpoint (**Table 2.20**).

Table 2.20 Daily intake of dihydrochalcones, as estimated by 4-DFD (n=161)

Polyphenols (mg/d)		**Control (n=82)**					**Dietary Guidelines (n=79)**				
Subclass	Individuals	Baseline		Endpoint		Change	Baseline		Endpoint		Change
Dihydrochalcones		1.0	(0.0, 2.8)	1.8	(0.0, 4.1)	0.2 (-0.8, 1.8)	1.5	(0.1, 3.4)	1.9	(0.5, 4.7)	0.0 (-1.5, 1.5)
	Phloretin	0.2	(0.0, 1.4)	0.7	(0.0, 2.0)	0.0 (-0.1, 0.9)	0.7[1]	(0.0, 1.8)	0.8	(0.1, 2.4)	0.0 (-1.0, 0.8)
	Phlorizin	0.7	(0.0, 1.4)	0.8	(0.0, 2.1)	0.1 (-0.4, 0.8)	0.8	(0.1, 1.7)	0.9	(0.4, 2.3)	0.0 (-0.6, 0.8)

All values are median (interquartile range)

[1]Mann-Whitney U test find difference between groups at baseline. There were no statistically significant differences between groups at endpoint, $P<0.05$.

There were statistically significant differences between groups in flavan-3-ols and (-)-epicatechin intakes at baseline, with higher intakes in the group randomised to the DG diet. However, there were no differences between groups for any of the individual compounds of the flavanols subclass at endpoint (**Table 2.21**).

Table 2.21 Daily intake of flavanols, as estimated by 4-DFD (n=161)

Polyphenols (mg/d)		**Control (n=82)**					**Dietary Guidelines (n=79)**				
Subclass	Individuals	Baseline		Endpoint		Change	Baseline		Endpoint		Change
Flavanols		272	(89, 488)	261	(99, 468)	1 (-48, 79)	367	(116, 530)	340	(135, 540)	-8 (-86, 69)
	Flavan-3-ols	205	(72, 355)	198	(81, 340)	2 (-10, 64)	275[1]	(86, 406)	257	(116, 394)	-8 (-63, 46)
	(+)-Catechin	33	(16, 55)	31	(17, 54)	1 (-10, 13)	41	(19, 65)	41	(14, 64)	-3 (-13, 8)
	(-)-Epicatechin	31	(16, 41)	32	(17, 44)	2 (-4, 10)	38[1]	(27, 52)	34	(20, 50)	-4 (-15, 5)
	(+)-Gallocatechin	44	(7, 88)	48	(9, 90)	0 (-8, 17)	59	(12, 105)	62	(11, 108)	0 (-16, 17)
	(-)-Epigallocatechin	27	(7, 49)	29	(13, 54)	0 (-5, 12)	38	(8, 64)	34	(13, 64)	0 (-12, 11)
	(-)-Epicatechin 3-O-gallate	25	(5, 49)	25	(8, 47)	0 (-4, 9)	36	(8, 53)	35	(13, 54)	0 (-9, 8)
	(-)-Epigallocatechin 3-O-gallate	35	(9, 63)	38	(16, 69)	0 (-6, 16)	50	(10, 81)	44	(18, 79)	0 (-16, 14)
	Theaflavins	36	(0, 75)	40	(0, 76)	0 (-7, 14)	50	(0, 89)	50	(0, 92)	0 (-16, 14)
	Theaflavin	10	(0, 20)	11	(0, 20)	0 (-2, 4)	13	(0, 23)	13	(0, 24)	0 (-4, 4)
	Theaflavin 3-O-gallate	5	(0, 9)	5	(0, 10)	0 (-1, 2)	6	(0, 11)	6	(0, 12)	0 (-2, 2)
	Theaflavin 3'-O-gallate	12	(0, 24)	13	(0, 25)	0 (-2, 5)	16	(0, 29)	16	(0, 30)	0 (-5, 5)
	Theaflavin 3,3'-O-digallate	10	(0, 21)	11	(0, 22)	0 (-2, 4)	14	(0, 25)	14	(0, 26)	0 (-4, 4)
	Other	22	(4, 49)	24	(6, 46)	0 (-6, 8)	31	(5, 54)	30	(4, 55)	-1 (-11, 8)

All values are median (interquartile range)

[1]Mann-Whitney U tests were carried out to find differences between groups at baseline and endpoint, there were differences between groups at baseline on catechins and epicatechin intakes. The difference was no longer significant between groups at endpoint, $P<0.05$

In the proanthocyanidins (PA) subclass there were statistically significant differences between groups in PA 1-3 mers, PA 4-6 mers, PA 7-10 mers and PA polymers intakes at baseline, with higher values in the group randomised to the DG diet. However, there were no differences between groups in PA 1-3 mers and PA 4-6 mers intakes at endpoint; and following Mann-Whitney U test for changes (endpoint – baseline) in both groups the difference was no longer significant in PA 7-10 mers and PA polymers intakes at endpoint (**Table 2.22**).

Table 2.22 Daily intake of proanthocyanidins, as estimated by 4DFD (n=161)

Polyphenols (mg/d)	**Control (n=82)**					**Dietary Guidelines (n=79)**				
Individuals	Baseline		Endpoint		Change	Baseline		Endpoint		Change
Proanthocyanidins (PA)	160	(77, 266)	187	(99, 271)	12 (-42, 98)	247[1]	(138, 337)	214	(127, 305)	10 (-108, 113)
Procyanidins	46	(19, 76)	43	(23, 71)	0 (-1, 14)	60	(33, 86)	54	(28, 91)	-5 (-22, 16)
Prodelphinidins	5	(1, 11)	5	(1, 10)	0 (-1, 1)	7	(1, 12)	7	(1, 12)	0 (-2, 3)
PA 1-3 mers	25	(10, 52)	31	(14, 63)	4 (-11, 25)	42[1]	(17, 72)	30	(15, 51)	-7 (-36, 15)
PA 4-6 mers	25	(9, 59)	27	(12, 57)	1 (-15, 25)	40[1]	(17, 86)	32	(18, 55)	-6 (-39, 18)
PA 7-10 mers	14	(5, 22)	17	(6, 27)	0 (-6, 11)	18[1]	(8, 34)	23	(15, 37)	3 (-6, 15)
PA polymers (>10 mers)	30	(13, 53)	39	(15, 66)	1 (-16, 25)	51[1]	(18, 90)	63	(42, 97)	14 (-21, 46)

All values are median (interquartile range)

[1]Mann-Whitney U tests were carried out to find differences between groups at baseline and endpoint, there were differences between groups at baseline. There were no statistically significant differences between groups at endpoint, $P<0.05$

There were no differences between groups for any of the individual compounds in the flavanones subclass at baseline, nevertheless there was a statistically significant difference between groups in eriodictyol intakes at endpoint, with higher values in the group following the DG diet (**Table 2.23**). In the DG diet at endpoint, lemons (52%), bitter orange marmalade (30%) and almonds (8%) were the main food sources for eriodictyol.

Table 2.23 Daily intake of flavanones, as estimated by 4-DFD (n=161)

Polyphenols (mg/d)		**Control (n=82)**			**Dietary Guidelines (n=79)**		
Subclass	Individuals	Baseline	Endpoint	Change	Baseline	Endpoint	Change
Flavanones		34 (5, 61)	24 (2, 51)	-1 (-22, 12)	23 (3, 58)	26 (7, 77)	3 (-14, 20)
	Naringenin	4 (0, 14)	3 (1, 10)	0 (-4, 1)	3 (1, 8)	5 (1, 12)	0 (-1, 5)
	Eriodictyol	0.002 (0, 0.020)	0.002 (0, 0.016)	0 (-0.01, 0)	0.003 (0, 0.060)	**0.022**[1] (0, 0.043)	0.01 (-0.02, 0.03)
	Hesperetin	15 (1, 32)	12 (1, 25)	0 (-13, 9)	11 (0, 32)	15 (3, 41)	3 (-8, 15)
	Other	4 (0, 15)	1 (0, 10)	0 (-5, 3)	2 (0, 9)	1 (0, 10)	0 (-5, 1)

All values are median (interquartile range)
[1]Mann-Whitney U tests were carried out to find differences between groups at baseline and endpoint, there were no differences between groups at baseline. There was a statistically significant difference between groups at endpoint, on eriodictyol intake, $P<0.05$.

There were no differences between groups for any of the individual compounds in the flavones subclass at baseline and endpoint (**Table 2.24**).

Table 2.24 Daily intake of flavones, as estimated by 4-DFD (n=161)

Polyphenols (mg/d)		**Control (n=82)**			**Dietary Guidelines (n=79)**		
Subclass	Individuals	Baseline	Endpoint	Change	Baseline	Endpoint	Change
Flavones		5 (3, 10)	5 (1, 12)	0 (-3, 4)	5 (2, 11)	4 (2, 10)	0 (-3, 3)
	Apigenin	2 (1, 5)	2 (0, 6)	0 (-2, 3)	3 (1, 6)	2 (0, 5)	0 (-2, 2)
	Luteolin	1 (0, 3)	1 (0, 2)	0 (-1, 0)	1 (0, 2)	1 (1, 3)	0 (-1, 0)

All values are median (interquartile range)
There were no differences between groups at baseline and endpoint, by Mann-Whitney U test, $P<0.05$

There were no differences between groups for any of the individual compounds in the flavonols subclass at baseline and endpoint (**Table 2.25**).

Table 2.25 Daily intake of flavonols, as estimated by 4-DFD (n=161)

Polyphenols (mg/d)		**Control (n=82)**					**Dietary Guidelines (n=79)**				
Subclass	Individuals	Baseline		Endpoint		Change	Baseline		Endpoint		Change
Flavonols		62	(40, 103)	68	(42, 100)	5 (-16, 21)	78	(45, 105)	75	(52, 117)	4 (-15, 24)
	Kaempferol	14	(5, 25)	16	(7, 26)	1 (-4, 6)	19	(7, 28)	18	(7, 32)	1 (-4, 6)
	Quercetin	42	(28, 64)	43	(27, 64)	1 (-12, 16)	48	(30, 68)	49	(31, 74)	3 (-8, 15)
	Myricetin	2	(1, 3)	1	(1, 4)	0 (-1, 1)	2	(1, 4)	2	(1, 4)	0 (-2, 2)
	Isorhamnetin	2	(1, 3)	1	(1, 3)	0 (-1, 1)	1[1]	(1, 2)	1	(1, 3)	0 (-1, 1)

All values are median (interquartile range)
[1]Mann-Whitney U tests were carried out to find differences between groups at baseline and endpoint, there were differences between groups at baseline on isorhamnetin intake. The difference was no longer significant between groups at endpoint, $P<0.05$

There were no differences between groups for any of the individual compounds in the isoflavones subclass at baseline. There were statistically significant differences between groups in daidzein and genistein intakes at endpoint, with greater intakes in the group following the DG diet (**Table 2.26**). In the DG group at endpoint the main food sources for daidzein were: soy drinks/fat free/fruit (62%), plain soy milk (17%), soy yogurt/fruit (16%) and soy and linseed bread (2%); for genistein main food sources were: soy drinks/fat free/fruit (66%), soy yogurt/fruit (16%), plain soy milk (12%) and soy and linseed bread (2%).

Table 2.26 Daily intake of isoflavones, as estimated by 4-DFD (n=161)

Polyphenols (mg/d)	**Control (n=82)**					**Dietary Guidelines (n=79)**				
Subclass Individuals	Baseline		Endpoint		Change	Baseline		Endpoint		Change
Isoflavones	0.26	(0.10, 6)	0.13	(0.06, 0.39)	-0.11 (-5, 0.06)	0.25	(0.13, 16.04)	**0.52**[1]	(0.06, 51)	0.05 (-0.21, 41)
Daidzein	0.09	(0.03, 2)	0.04	(0.02, 0.12)	-0.06 (-1, 0.02)	0.09	(0.04, 6.57)	**0.11**[1]	(0.01, 16)	0.03 (-0.06, 13)
Genistein	0.14	(0.05, 3)	0.08	(0.04, 0.28)	-0.06 (-3, 0.05)	0.16	(0.09, 8.23)	**0.47**[1]	(0.03, 31)	0.02 (-0.13, 24)

All values are median (interquartile range)
[1]Mann-Whitney U tests were carried out to find differences between groups at baseline and endpoint, there were no differences between groups at baseline. There were statistically significant differences between groups at endpoint, $P<0.05$.

There were no differences between groups for any of the individual compounds in the lignans subclass at baseline. There were statistically significant differences between groups for secoisolariciresinol and matairesinol intakes at endpoint. Higher intakes were observed in the group following the DG diet (**Table 2.27**). Linseeds (94%), black tea (3.5%), brown bread (0.9%) and crispbread rye (0.7%) were the main food sources for secoisolariciresinol and linseeds (56%), black tea (23%), muesli, luxury fruit and nut (7%), and sunflower seeds (3.5%) were the major food sources for matairesinol.

Table 2.27 Daily intake of lignans, as estimated by 4-DFD (n=161)

Polyphenols (mg/d)	**Control (n=82)**					**Dietary Guidelines (n=79)**				
Subclass Individuals	Baseline		Endpoint		Change	Baseline		Endpoint		Change
Lignans	0.34	(0.18, 2.45)	0.26	(0.13, 0.44)	-0.05 (-0.42, 0.07)	0.52	(0.21, 4.43)	**0.42**[1]	(0.20, 5)	0.03 (-0.37. 2)
Pinoresinol	0.05	(0.02, 0.11)	0.02	(0.01, 0.04)	-0.02 (-0.07, 0.01)	0.07	(0.02, 0.15)	0.01	(0, 0.03)	- 0.04 (-0.11, 0.01)
Lariciresinol	0.02	(0.01, 0.05)	0.02	(0.01, 0.04)	0 (-0.02, 0.02)	0.03	(0.01, 0.05)	0.03	(0.01, 0.06)	0 (-0.02, 0.02)
Secoisolariciresinol	0.16	(0.05, 0.30)	0.14	(0.06, 0.26)	0 (-0.04, 0.04)	0.19	(0.10, 0.30)	**0.18**[1]	(0.10, 0.38)	0.01 (-0.08, 0.09)
Matairesinol	0.01	(0.01, 0.02)	0.01	(0.01, 0.02)	0 (-0.01, 0.01)	0.02	(0.01, 0.03)	**0.02**[1]	(0.01, 0.03)	0 (0, 0.01)

All values are median (interquartile range)
[1]Mann-Whitney U tests were carried out to find differences between groups at baseline and endpoint, there were no differences between groups at baseline. There were statistically significant differences between groups at endpoint, $P<0.05$.

There were statistically significant differences between groups for 4-hydroxybenzoic acid and p-coumaric acid intakes at baseline, with higher intakes in the group randomised to the DG diet. However, following Mann-Whitney U test for changes (endpoint – baseline) in both groups the difference was no longer significant at endpoint. There were no differences between groups for any other individual compound in the phenolic acids subclass at baseline. There were statistically significant differences between groups for protocatechuic acid, vanillic acid and syringic acid intakes at endpoint. Greater values were observed in the group following the DG diet (**Table 2.28**). The main food sources, in DG group at endpoint, for protocatechuic acid were: apples (16%), strawberries (15%) and white wine (12%); for vanillic acid were: dates (17%), beer (lager) (10%) and muesli, luxury fruit and nuts (9%); and for syringic acid were: apples (26%), Swiss chard (17%) and hummus (16%).

Table 2.28 Daily intake of phenolic acids, as estimated by 4-DFD (n=161)

Polyphenols (mg/d)	**Control (n=82)**					**Dietary Guidelines (n=79)**				
Subclass Individuals	Baseline		Endpoint		Change	Baseline		Endpoint		Change
Phenolic acids	228	(121, 502)	217	(114, 467)	3 (-83. 53)	248	(164, 576)	**300**[1]	(155, 554)	3 (-52, 71)
Hydroxybenzoic acids	63	(27, 120)	68	(32, 117)	-3 (-20, 24)	95	(42, 140)	87	(40, 139)	0 (-31, 24)
Protocatechuic acid	0.8	(0.5, 1)	0.8	(0.6, 1)	0.1 (-0.5, 0.4)	1	(0.6, 2)	**1**[1]	(0.7, 2)	0.2 (-0.5, 0.6)
Gallic acid	52	(10, 108)	56	(20, 103)	0 (-11, 19)	70	(18, 119)	72	(20, 121)	0 (-22, 19)
Vanillic acid	0.2	(0.1, 0.4)	0.2	(0.1, 0.3)	0 (-0.1, 0.1)	0.3	(0.1, 0.5)	**0.3**[1]	(0.2, 0.5)	0.1 (0, 0.2)
4-Hydroxybenzoic acid	2	(1, 3)	2	(1, 3)	0 (-2, 1)	2[1]	(1, 5)	3	(1, 6)	1 (-1, 2)
Syringic acid	0.5	(0.2, 1)	0.4	(0.2, 1)	0.2 (-0.4, 0.5)	0.6	(0.3, 1)	**0.9**[1]	(0.5, 2)	0.2 (-0.3, 0.8)
Other	6	(1, 14)	4	(1, 8)	-1 (-8, 3)	6	(2, 15)	4	(2, 11)	0 (-7, 5)
Hydroxycinnamic acids	131	(67, 406)	117	(56, 406)	1 (-89, 41)	129	(84, 455)	147	(83, 425)	12 (-59, 84)
p-Coumaric acid	8	(4, 13)	9	(5, 14)	1 (-2, 2)	11[1]	(7, 15)	12	(8, 17)	2 (-1, 5)
Caffeic acid	70	(27, 325)	75	(33, 333)	3 (-43, 48)	74	(43, 374)	86	(49, 359)	10 (-39, 76)
Ferulic acid	23	(9, 56)	13	(6, 53)	-4 (-19, 5)	24	(6, 59)	23	(8, 57)	1 (-14, 15)
Other	13	(5, 31)	7	(3, 24)	-1 (-9, 6)	9	(3, 36)	11	(4, 25)	0 (-9, 6)

All values are median (interquartile range)

[1]Mann-Whitney U tests were carried out to find differences between groups at baseline and endpoint, there were differences between groups at baseline on 4-Hydroxybenzoic acid intakes. There were statistically significant differences between groups at endpoint, $P<0.05$.

There were no differences between groups for any of the individual compounds in the stilbenes subclass at baseline and endpoint (**Table 2.29**)

Table 2.29 Daily intake of stilbenes, as estimated by 4-DFD (n=161)

Polyphenols (mg/d)		**Control (n=82)**					**Dietary Guidelines (n=79)**				
Subclass	Individuals	Baseline		Endpoint		Change	Baseline		Endpoint		Change
Stilbenes		0.05	(0.01, 0.54)	0.09	(0.02, 0.85)	0 (-0.07, 0.16)	0.11	(0.02, 0.73)	0.19	(0.05, 1.00)	0.03 (-0.10, 0.34)
	Resveratrol	0.05	(0.01, 0.34)	0.09	(0.02, 0.54)	0 (-0.06, 0.14)	0.11	(0.02, 0.45)	0.19	(0.04, 0.65)	0.02 (-0.07, 0.24)

All values are median (interquartile range)
There was no difference between groups at baseline and endpoint, by Mann-Whitney U test, $P<0.05$

When comparing results obtained from the EPIC-Norfolk FFQ and 4-DFD it was found that FFQ report more changes at endpoint in dietary flavonoid intakes than 4-DFD did, more subclasses and individual compounds showed significant higher intakes in the intervention group when estimated by FFQ (**Table 2.30**). However FFQ and 4-DFD agree in results derived from anthocyanidins; cyanidin, malvidin and peonidin intakes. It can be also noted that 4-DFD is more sensitive in estimating the different compounds analysed, since it was able to detect higher values of intakes in most of the flavonoids analysed. Worth to mention is the fact that the database used for the FFQs analysis estimates only flavonoid intakes while the database for 4-DFD analysis contemplated a more complete range of polyphenols.

Table 2.30 Flavonoid intakes in the **Dietary Guidelines** intervention group at endpoint and comparison with control group

Flavonoids (mg/d)		**Intakes at endpoint**			
Subclasses	**Individuals**	**FFQ**		**4-DFD**	
Total flavonoids		**714**	>	788	–
Anthocyanidins		**32**	>	69	–
	Cyanidin	**15**	>	**7**	>
	Malvidin	**8**	>	**11**	>
	Peonidin	**0.7**	>	**2**	>
	Pelargonidin	**5**	>	3	–
	Delphinidin	**2**	>	4	–
Flavones		**3**	>	4	–
	Luteolin	**2**	>	1	–
Flavan-3-ols		**97**	>	257	–
	(+)-Catechin	**15**	>	41	–
	(-)-Epicatechin 3-O-gallate	**15**	>	35	–
	(-)-Epigallocatechin 3-O-gallate	**24**	>	44	–
Flavonols		39	–	75	–
	Myricetin	**2**	>	2	–
Flavanones		27	–	26	–
	Eriodictyol	0.01	–	**0.02**	>
Isoflavones		NE		**0.52**	>
	Daidzein	NE		**0.11**	>
	Genistein	NE		**0.47**	>
Polymeric flavonoids		**496**[1]	>	268[2]	–
	Proanthocyanidins (PA)	**290**	>	214	–
	PA polymers (>10 mers)	**85**	>	63	–

All values are median
> Greater at endpoint relative to control; – not greater at endpoint relative to control group, NE not estimated; PA, proanthocyanidins.
[1]Theaflavins, thearubigins and PA
[2]Theaflavins and PA

2.4.2.1 Relationships between dietary polyphenol intakes estimated by 4-DFD and markers of glucose homeostasis

In a correlation analysis, higher intake of the lignan, pinoresinol, was associated with lower fasting blood glucose levels. Major food sources of lignans in the entire population of CRESSIDA study were linseeds (40%), olive oil (9%) and granary bread (6%). Flavonoids subclasses, proanthocyanidins, flavanones and flavones, and the phenolic acid, ferulic acid, were associated with lower insulin levels. Main food sources for proanthocyanidins were black tea (20%), apples (19%), plain chocolate (10%) and strawberries (5%); for flavanones, orange juice (31%), oranges (23%) and fruit drinks (22%); for flavones, herbal tea (33%), orange juice (23%) and granary bread (7%); and for ferulic acid, coffee (59%), cous cous (9%) and soft wholemeal bread (4%). Following the tendency, flavanones, flavones and ferulic acid were significantly associated with lower values of HOMA-IR, an index used to estimate insulin resistance. Delphinidin was significantly associated with lower insulin values and higher values on RQUICKI, an index used to estimate insulin sensitivity. Major food sources of delphinidin were concentrated fruit drinks (41%), concentrated blackcurrant juice (27%) and red wine (9%). No other significant correlations were noted for other polyphenol classes, subclasses or individual polyphenols. See **Table 2.31** for correlation coefficients values.

Table 2.31 Spearman's correlation coefficients between biomarkers of glucose homeostasis and dietary intake of polyphenols at endpoint (n=161)

Polyphenols (mg/d)	Pinoresinol		Proanthocyanidins		Flavanones		Flavones		Ferulic acid		Delphinidin	
Biomarker	*r*	P value	*r*	P value	*r*	P value	*r*	P value	*r*	P value	*r*	P value
Glucose (mg/dl)	**-0.184**	0.020	0.000	0.999	0.017	0.827	-0.003	0.970	0.007	0.931	-0.005	0.950
Insulin (mU/L)	-0.037	0.641	**-0.162**	0.040	**-0.232**	0.003	**-0.188**	0.017	**-0.190**	0.016	**-0.104**	0.019
HOMA-IR	-0.065	0.410	-0.153	0.052	**-0.221**	0.005	**-0.190**	0.016	**-0.183**	0.020	-0.101	0.203
RQUICKI	0.103	0.194	0.045	0.567	**0.211**	0.007	**0.175**	0.026	0.096	0.226	**0.181**	0.021

r; Spearman's correlations coefficient, *P* is significant at 0.05 level (2-tailed)
HOMA-IR; homeostatic model assessment of insulin resistance, RQUICKI; revised quantitative insulin sensitivity check index

2.4.3 Urinary polyphenol excretion by Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS)

In order to provide a more objective estimate of polyphenol intake, urinary analysis of ten selected aglycones was carried out on a subsample, as described in section **2.3.5**. Descriptive data of the CRESSIDA study subsample at baseline is shown in **Table 2.32**, demonstrating that the sub sample was similar in age, BMI and gender distribution to the whole cohort daily polyphenol intake was estimated from the 4-DFD. Similarly to the total population the major contributors of polyphenol intake in this subsample, are represented by the subclasses; flavonoids and phenolic acids followed by stilbenes and lignans.

Table 2.32 Characteristics of the CRESSIDA study subsample (n=91) selected for urinary analysis compared to the total study cohort who completed the intervention (n=161)

Characteristic	**Endpoint** (n=91)	**Endpoint** (n=161)
Gender (F/M)	54/37	97/64
Age[1] (years)	54 ± 8	54 ± 8
BMI[1] (kg/m^2)	25.4 ± 3.9	26.0 ± 3.9
Total polyphenols[2] (mg/d)	1178 (928)	1124 (924)
Flavanoids[2] (mg/d)	742 (797)	727 (623)
Lignans[2] (mg/d)	0.3 (0.5)	0.3 (0.5)
Phenolic acids[2] (mg/d)	253 (428)	239 (408)
Stilbenes[2] (mg/d)	0.26 (0.96)	0.15 (0.91)

[1] Values are mean ± SD
[2] Values are median (interquartile range), estimated by 4-DFD

Data on the excretion of the ten phenolic compounds quantified are displayed in **Table 2.33**; the highest quantities detected in the 24 h urine samples were for enterolactone followed by quercetin whereas lowest quantities were for phloretin and daidzein.

Table 2.33 Excretion of polyphenols in 24 h urine in a subsample of the CRESSIDA study population (n=91)

Polyphenols	**Endpoint**[1] (mg/g creatinine)
Phloretin	0.04 (0.12)
Epicatechin	0.10 (0.11)
Eriodictyol	0.08 (0.16)
Hesperetin	0.13 (0.68)
Luteolin	0.53 (0.55)
Quercetin	1.26 (2.13)
Daidzein	0.01 (0.09)
Gallic acid	0.13 (0.35)
Vanillic acid	0.26 (0.42)
Enterolactone	1.73 (3.09)

[1]All values are median (IQR)

The aglycones quantified are either metabolites of parent glucuronide or sulphated compounds formed in human tissues, or metabolites formed by the gut microbiota, e.g. enterolactone from lignans present in fibre-rich food. There were statistically significant differences between groups for phloretin, eriodictyol, hesperetin, luteolin, quercetin, gallic acid, vanillic acid and enterolactone with higher excretion in the group following the DG diet (**Table 2.34**). There were no differences between groups for epicatechin and daidzein at endpoint. Although data suggests a tendency in daidzein excretion, there was no statistical significant difference between groups. Daidzein was not detected in 37 participants of the 91 subsample; this could contribute to the lack of significance in the results given that values observed are considerably higher in the intervention group. The data on urinary excretion is presented along with data in dietary intake of the same aglycone or the direct dietary precursor.

Table 2.34 Excretion of polyphenols in 24 h urine in a subsample of the CRESSIDA study population and dietary intake of the direct dietary precursor estimated by 4-DFD (n=91)

Polyphenol	**Control** (n=46)		**Dietary Guidelines** (n=45)		*P* value
	Dietary intake (mg/d)	**Urinary excretion**[1] (mg/g creatinine)	**Dietary intake** (mg/d)	**Urinary excretion**[1] (mg/g creatinine)	
Phloretin	0.5 (0.0 – 1.4)	0.02 (0.00, 0.04)	1.3[2] (0.3 – 2.7)	**0.09**[2] (0.05, 0.18)	0.000
Epicatechin	28 (14 – 46)	0.10 (0.05, 0.15)	38 (19 – 52)	0.09 (0.05, 0.18)	0.557
Eriodictyol	0.00 (0.00 – 0.02)	0.05 (0.02, 0.09)	0.02[2] (0.01 – 0.05)	**0.12**[2] (0.05, 0.23)	0.005
Hesperetin	6 (1 – 22)	0.04 (0.00, 0. 34)	15[2] (4 – 32)	**0.44**[2] (0.02, 0.98)	0.005
Luteolin	0.4 (0.3 – 0.8)	0.34 (0.20, 0.64)	1.1[2] (0.7 – 3.0)	**0.70**[2] (0.35, 0.92)	0.004
Quercetin	38 (24 – 69)	0.94 (0.41, 1.97)	56 (34 – 74)	**1.80**[2] (0.61, 3.35)	0.038
Daidzein	0.03 (0.02 – 1)	0.00 (0.00, 0.07)	3.6[2] (0.0 – 19)	0.04 (0.00, 0.20)	0.065
Gallic acid	54 (19 – 108)	0.09 (0.02, 0.25)	70 (19 – 117)	**0.21**[2] (0.07, 0.61)	0.026
Vanillic acid	0.1 (0.1 – 0.2)	0.23 (0.03, 0.44)	0.3[2] (0.2 – 0.5)	**0.50**[2] (0.15, 0.99)	0.010
Lignan/Enterolactone[3]	0.2 (0.1 – 0.4)	1.71 (0.64, 3.16)	0.4[2] (0.2 – 5.4)	**3.03**[2] (1.15, 6.74)	0.001

All values are median (interquartile range)
[1] Values adjusted for 24 h urine volume and g of creatinine
[2] Mann-Whitney U tests were carried out to find differences between groups at endpoint, there were statistically significant differences between groups in dietary intakes and urinary excretion of polyphenols, with higher values in group following the intervention diet, $P<0.05$.
[3] Intake of lignans was used as direct dietary precursor of enterolactone

Urinary excretion of polyphenols in 24 h samples was correlated with dietary intakes estimated by 4-DFD in the whole subsample (n=91). Most of the individual polyphenols quantified have a significant and positive correlation with intake of their direct precursor. There was a strong and positive correlation between urinary excretion and estimated dietary intake of gallic acid (r = 0.730, $P < 0.001$) and phloretin (r =0.472, $P < 0.001$). There was a weaker correlation between urinary excretion and estimated dietary intake of epicatechin, hesperetin, daidzein and enterolactone (r_s between 0.235 and 0.375, $P < 0.05$). There was no significant correlation between urinary excretion and estimated dietary intake of eriodictyol, luteolin, quercetin and vanillic acid (**Table 2.35**).

Table 2.35 Spearman's correlations coefficients between urinary excretion of polyphenols in 24 h urine samples and dietary intake of direct dietary precursors estimated by 4-DFD (n=91)

Polyphenols Urinary	**Phloretin**		**Epicatechin**		**Eriodictyol**		**Hesperetin**		**Luteolin**	
Dietary	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value
Subclass Individual										
Dihydrochalcones	**0.506**	0.000	**0.385**	0.000	0.159	0.135	0.110	0.301	0.123	0.248
Phloretin	**0.472**	0.000	**0.407**	0.000	0.158	0.138	0.109	0.306	0.109	0.306
Phlorizin	**0.512**	0.000	**0.385**	0.000	0.165	0.119	0.112	0.291	0.140	0.183
Flavan-3-ols	0.092	0.390	0.207	0.050	0.207	0.050	-0.145	0.173	**0.279**	0.008
Epicatechin	0.151	0.155	**0.360**	0.000	**0.360**	0.000	-0.177	0.095	**0.271**	0.010
Flavanones	**0.237**	0.025	0.014	0.897	0.193	0.068	**0.217**	0.040	0.050	0.637
Naringenin	**0.318**	0.002	**0.212**	0.044	**0.277**	0.008	**0.213**	0.043	0.178	0.093
Eriodictyol	0.124	0.244	0.053	0.620	0.002	0.982	-0.114	0.284	0.123	0.250
Hesperetin	**0.245**	0.020	-0.006	0.956	**0.228**	0.031	**0.258**	0.014	0.065	0.540
Flavones	0.161	0.129	0.083	0.438	0.095	0.373	0.059	0.579	0.003	0.778
Luteolin	**0.306**	0.003	**0.228**	0.031	0.036	0.739	0.105	0.324	0.108	0.311

Table 2.35 continued

Polyphenols Urinary	**Quercetin**		**Daidzein**		**Gallic acid**		**Vanillic acid**		**Enterolactone**	
Dietary	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value
Subclass Individual										
Flavonols	0.199	0.060	-0.121	0.254	**0.498**	0.000	**0.209**	0.049	0.184	0.083
Kaempferol	**0.263**	0.012	-0.068	0.523	**0.688**	0.000	**0.309**	0.003	**0.217**	0.040
Quercetin	0.194	0.067	-0.100	0.351	**0.493**	0.000	0.164	0.123	0.114	0.283
Myricetin	**0.260**	0.013	0.122	0.253	0.139	0.193	0.077	0.471	**0.302**	0.004
Isoflavones	-0.007	0.949	**0.354**	0.000	-0.109	0.307	0.161	0.129	0.056	0.603
Daidzein	-0.029	0.787	**0.375**	0.000	-0.127	0.234	0.138	0.196	0.070	0.514
Genistein	0.006	0.953	**0.367**	0.000	-0.105	0.324	0.152	0.325	0.051	0.634
Phenolic acids	**0.208**	0.049	-0.033	0.757	**0.253**	0.016	**0.226**	0.032	**0.241**	0.022
Gallic acid	**0.247**	0.019	-0.036	0.737	**0.730**	0.000	**0.284**	0.007	0.162	0.126
Vanillic acid	0.159	0.135	-0.040	0.706	0.010	0.925	0.034	0.753	0.092	0.386
Lignans	**0.317**	0.002	0.124	0.245	**0.352**	0.001	**0.306**	0.003	**0.311**	0.003
Secoisolariciresinol	**0.270**	0.010	-0.147	0.167	**0.609**	0.000	**0.239**	0.023	**0.235**	0.026
Matairesinol	**0.323**	0.002	-0.019	0.856	**0.553**	0.000	**0.216**	0.041	**0.347**	0.001

r; Spearman's correlation coefficient, *P* is significant at the 0.05 level (2-tailed)

2.4.3.1 Relationships between urinary excretion of polyphenols estimated by LC-MS/MS and markers of glucose homeostasis

In a correlation analysis, higher excretion of the phenolic compounds; enterolactone, eriodictyol, hesperetin, phloretin and quercetin were associated with biomarkers of glucose homeostasis and cardiovascular disease risk (vascular function and blood pressure). Higher excretion of enterolactone was associated with lower values of insulin and HOMA-IR. Higher excretion of eriodictyol, hesperetin and phloretin were associated with lower values for pulse wave reflection index (higher reflection index indicates increased systemic vascular resistance). Hesperetin showed an inverse association with 24 h systolic and diastolic blood pressure as well as quercetin with 24 h diastolic blood pressure (**Table 2.36**).

Table 2.36 Spearman's correlation coefficients between urinary excretion of polyphenols in 24 h urine samples and biomarkers of glucose homeostasis and vascular function (n=91)

Polyphenol	**Enterolactone**		**Eriodictyol**		**Hesperetin**		**Phloretin**		**Quercetin**	
Biomarker	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value
Insulin (mU/L)	**-0.214**	0.042	-0.118	0.267	-0.067	0.541	-0.001	0.990	-0.057	0.593
HOMA-IR	**-0.215**	0.041	-0.090	0.399	-0.041	0.699	0.031	0.772	-0.056	0.595
Reflection index (%)	-0.053	0.633	**-0.237**	0.030	**-0.241**	0.027	**-0.236**	0.031	-0.085	0.443
24 h systolic blood pressure (mmHg)	-0.057	0.591	-0.145	0.184	**-0.227**	0.031	-0.081	0.445	-0.076	0.473
24 h diastolic blood pressure (mmHg)	-0.013	0.210	-0.086	0.419	**-0.271**	0.009	-0.129	0.223	**-0.234**	0.026

r; Spearman's correlations coefficient, *P* is significant at 0.05 level (2-tailed)
HOMA-IR; homeostatic model assessment of insulin resistance

2.5 Discussion

In the present study we estimated dietary intake of total polyphenols, polyphenol subclasses and individual polyphenols on the CRESSIDA population using two dietary methods; FFQ and 4-DFD and one biomarker approach; urinary excretion estimated by LC-MS/MS. Total intake of 52 polyphenol aglycones were estimated from dietary records and excretion of ten polyphenol aglycones from 24 h urine samples, major food sources were also identified. To our knowledge this is the first study using simultaneously three different approaches for dietary assessment in a randomised controlled trial. Compliance to dietary advice was evaluated and reported in the main CRESSIDA report (Reidlinger *et al.,* 2015). Self-reported average intakes of fruit and vegetables were of 6 portions/d according to the FFQ and slightly less than 5 portions/d in the 4-DFD. Urinary potassium excretion was 9 mmol/d greater in the intervention group indicating higher consumption of fruit and vegetables by the participants. Dietary fiber intakes at baseline were higher than those reported in the National Diet and Nutritional Survey (24 g/d compared with 20 g/d). Whole-grain intake increased to 81 g/d in the intervention group compared with 32 g/d in control group, main food sources were wheat, oats, and rice, therefore dietary fiber intake was 7 g/d higher in the invertion group, reflecting increased cereal fiber from whole grains and confirmed by higher plasma alkylresorcinol concentrations, which reflect intakes of whole grains mainly from wheat, barley, and rye but not rice or oats.

Our polyphenol intake estimates are in agreement with other reports in UK populations using 24-HR (Zamora-Ros *et al.,* 2011a, Zamora-Ros *et al.,* 2011b, Vogiatzoglou *et al.,* 2013, Zamora-Ros *et al.,* 2015) or FFQs (Cassidy *et al.,* 2011, Jennings *et al.,* 2012). Most remarkable findings were increased intakes of individual polyphenols which main food sources were fruits (berries, citrus, apples and bananas), vegetables (sweet peppers and lettuce), nuts (peanuts, mixed nuts), seeds (linseeds, sesame seeds) and soy products (soy drinks and soy milk).

Results demonstrate that following advice to conform to current UK dietary guidelines, compared to the average UK dietary pattern, does result in a higher intake of specific polyphenols that may confer cardiometabolic protective effects. This study reinforces the finding from previous report (Reidlinger *et al.,* 2015) that providing advice to follow

UK dietary guidelines effectively increases the consumption of wholegrains, fruits and vegetables. CRESSIDA (ISRCTN92382106).

2.5.1 FFQ

Dietary intake of six flavonoid subclasses and 32 aglycone polyphenols were estimated by FFQ and main food sources were also identified. Consumption of flavonoids; anthocyanidins, flavones, flavan-3-ols and polymeric flavonoids increased in participants of the CRESSIDA study following a DG diet. FFQ methodology indicated increased intakes of 12 individual aglycone polyphenols in the CRESSIDA population following the DG diet. Differences in polyphenol intakes where major food sources were fruit and vegetables accounted for most of the changes detected on the flavonoid subclasses, anthocyanidins represented by consumption of berries (strawberries, raspberries and kiwi fruit), flavones for citrus fruit (oranges, satsumas and mandarins) and proanthocyanidins for apples. Increased estimated intakes of individual compounds such as myricetin, flavan-3-ols; epicathechin-3-gallate and epigallocatechin-3-O-gallate and polymeric flavonoids were found in the group following the DG diet. Although intakes of those particular flavan-3-ols mainly represent consumption of black tea in this population analysis of reported tea-drinking discards more tea-drinking at endpoint. Secondary food sources analysis indicates that the increases correspond to a greater intake of grapes and stone fruits (peaches, plums and apricots) for epicathechin-3-gallate and berries, stone fruits and pears for epigallocatechin-3-O-gallate.

Although the FFQ is a valid and reliable dietary assessment tool, it has limitations in the way that it can collect information, since is based on estimated portion sizes and is semi-quantitative on its answers. The EPIC-Norfolk FFQ although validated for whole food intake, validation for flavonoid intake has not been reported (McKeown *et al.*, 2001). The method has some limitations in regard to flavonoid estimation since some foods have been grouped incongruously with their flavonoid composition such as strawberries, raspberries and kiwi, or peaches, plums and apricots and red and white wine. Some important flavonoid sources are missing such as blueberries, soy beverages, and lemon, among others. The inadequacy of the method could lead to under or overestimation of flavonoid intakes. More specialised questionnaires for intake estimation of individual compounds like quercetin and naringenin (Ranka *et al.*, 2008), flavonoid subclasses such as flavonols and flavones in Chinese population (Zhang *et al.*, 2010), complete flavonoid class in Australian population (Somerset and Papier, 2014) and Flemish population (Mullie *et al.*, 2007) have been developed and used in diverse

studies. The database created for FFQs analysis might also add some limitations in the flavonoid intake estimation, since it was developed in 2012 before the release of the latest update of the flavonoid USDA composition food table and some values might be inaccurate, due to methodological aspects in the quantification. In addition the USDA composition food tables do not include retention factors that allow the adjustment of polyphenol food content after cooking process. Standardisation of a food frequency questionnaire for flavonoid and/or polyphenol intake and agreement in the polyphenol database used for estimation might improve intake estimation and facilitate comparison between populations.

2.5.2 4-DFD

Dietary intake of flavonoids, lignans, phenolic acids, stilbenes and other polyphenol subclasses was estimated by 4-DFD. Total intake of 52 polyphenol aglycones were estimated and main food sources were identified. Consumption of lignans, phenolic acids and other polyphenols increased in participants of the CRESSIDA study following the DG diet. 4-DFD indicated increased intakes of 11 out of 52 individual aglycone polyphenols in the CRESSIDA population following the DG diet. Differences in polyphenol intakes occurred for subclasses mainly where major food sources were fruit and vegetables. For example, anthocyanidins represented by consumption of berries and berry beverages (raspberries, red wine, grapes and blackcurrant juice), eriodictyol for citrus fruit and citrus jams (lemon and orange marmalade), isoflavones for soy beverages (soy drinks and soy milk), lignans by seeds (linseeds), phenolic acids by apples and dates and proanthocyanidins by apples. Where there were no differences in polyphenols they were provided mainly by food sources representative of the UK population diet, like black tea. This discrimination of the 4-DFD is in agreement with the aim of the intervention of increasing intakes of fruit and vegetables instead of modifying representative dietary patterns.

The 4-DFD is a more accurate tool for the quantification of food portions consumed but is more of a burden on the study participants to complete it accurately, and only represents eating habits during those four days (which include one weekend day), thereby missing or overstimating foods that might be consumed seasonally or infrequently. The main advantage of this method is the description and quantification of all foods consumed in the four-day period. The detailed and specific analysis of all foods must account for a big part on the differences between results from FFQ and 4-DFD. In addition the use of different food composition tables used, USDA for the FFQ

analysis and Phenol-Explorer/USDA for the 4-DFD analysis might explain as well some of the discrepancies in results (Peterson *et al.*, 2015). In a separate study comparison between FFQ and 7-DFD have showed that food diaries provided a better estimation of nutrient intakes than FFQ (Day *et al.*, 2001), although could be an overwhelming method for participants and expensive for its use on a big population study.

Some studies have used food diaries for estimation of flavanol intake in European population (Vogiatzoglou *et al.*, 2013) and isoflavone and lignan intakes (Mulligan *et al.*, 2013) and 20 subclasses of polyphenols (Yahya *et al.*, 2015) in UK population, each one creating their own database for polyphenol analysis. The database developed for the 4-DFD polyphenol analysis in the present study included the estimation of 52 individual compounds of the different four polyphenol classes, and contained 1141 foods representative of an average UK diet and some of the most commonly eaten food from different cuisine as Turkish, Indian, Italian, Chinese, Japanese, among other. This database could be used for future studies analysing diet patterns in the UK population, comparing polyphenols intake and/or linking intake with health benefits.

2.5.3 LC-MS/MS

Urinary excretion of ten representative aglycone polyphenols were quantified in 24 h urine samples by LC-MS/MS. Urinary excretion of phloretin, eriodictyol, hesperetin, luteolin, vanillic acid and enterolactone increased in the CRESSIDA study subsample following the DG diet. Increases in urinary excretion mirror some of the findings in dietary intake analyis, however LC-MS/MS detected some changes not reported by FFQ and 4-DFD. Differing findings may be result of the high specificity and sensitivity of the LC-MS/MS method (Nielsen *et al.*, 2000) or due to the nature of dietary methods where inaccuracies can be introduced by self-reported intakes, portion size estimation, food classification and food composition tables used (Bingham *et al.*, 1994, Zamora-Ros *et al.*, 2012).

The analysis by dietary methods may not be sensitive enough to detect changes in phloretin intakes, as LC-MS/MS analysis did, but was able to detect increases in apple intakes in the DG group at endpoint. Apple is the main food source of phloretin and both dietary methods reported higher intakes in polyphenols for which main food sources were apples, in FFQ there was an increase in proanthocyanidins intakes and in 4-DFD there were increases in protocatechuic acid and syringic acid intakes. However, high phloretin urine concentrations could also be result of endogenous metabolism of

naringenin to phloretin (Ito *et al.*, 2005). Besides apples, strawberries and white wine were the main food sources of protocatechuic acid and Swiss chard and hummus of syringic acid. Vanillic acid can be find in high concentrations in foods consumed in small quantities as herbs, basil, thyme and oregano; also in moderate concentration in fruits as dates and cranberries and in lower quantities in foods more frequently consumed as oat and rice (Rothwell *et al.*, 2013). It is also a common metabolite of anthocyanins (Nurmi *et al.*, 2009, de Ferrars *et al.*, 2013), therefore increased urinary excretion could reflect increased intakes of vanillic acid per se as well as parent metabolites such as anthocyanins. Vanillic acid has also showed to be present in urine after consumption of characteristic UK diet foods as black tea (Olthof *et al.*, 2003) and dark chocolate (Rios *et al.*, 2003) which did not increase at endpoint in the CRESSIDA population, therefore are unlikely to account for the increased urinary excretion. However correlation analysis did not show a statistically significant association between dietary intakes of vanillic acid and urinary excretion therefore is likely that the increased value at endpoint reflect intake of parent compounds of vanillic acid.

Enterolactone is the main colonic metabolite of lignans, phenolic compounds present in high concentrations in seeds, nuts and berries (Patel *et al.*, 2012). FFQ and 4-DFD reported increases at endpoint in polyphenols which main food sources were seeds (lignans), nuts (other polyphenols) and berries (anthocyanidins) and LC-MS/MS detected increases in enterolactone excretion. Enterolactone has also showed an increase excretion in urine after supplementation with soy protein (Xu *et al.*, 2000), consumption of soy-containing food (Rowland *et al.*, 2000) and whole grain cereals (Johnsen *et al.*, 2004), so increases are likely to be related to the content of lignans in the food. 4-DFD reported increases in isoflavones intakes at endpoint in the intervention group; therefore increase in enterolactone excretion, as a microbiota metabolite, might reflect dietary intakes of diverse polyphenols subclasses. Our results are in agreement with previous studies associating polyphenol dietary intakes and urinary excretion in epidemiologial studies (Valls-Pedret *et al.*, 2012), supplementation studies where known doses of polyphenols were administrated (Shi and Williamson, 2015, Perez-Jimenez *et al.*, 2010a), in RCTs aiming to increase flavonid-rich fruit and vegetables intake (Chong *et al.*, 2012) and with population under free-living conditions (Mennen *et al.*, 2008). Polyphenol urinary excretion has proved to be an efficient biomarker of fruit and vegetables intakes, capable of detecting even small changes in studies under controlled

diets (Nielsen *et al.*, 2002, Brevik *et al.*, 2004) but no similar analysis has been done in a RCTs with a free-living population following advice to adhere to dietary guidelines.

Limitation of the urinary excretion analysis is that according to dietary assessment methods there were differences at baseline intakes but we did not measure baseline values in urine excretion to verify this. The LC-MS/MS quantification of urine polyphenols eliminate the inaccuracies of the dietary methods but, since urinary excretion of polyphenols depends on intake and bioavailability, results may reflect interindividual variability in absorption and metabolism (Chen *et al.*, 2014, Rodriguez-Mateos *et al.*, 2014) and not only dietary intakes, moreover the method required the preselection of polyphenols to be analysed which means that many other polyphenols present in 24 h urine that are not detected and quantified could function as biomarkers of intake. Relevant polyphenols that were not analysed include anthocyanins, which have showed low recovery percentages and weak correlation with dose ingested (Perez-Jimenez *et al.*, 2010). The use of a high sensitive method like LC-MS/MS represents a high cost for the researcher and is not always practical when a large population is involved.

Although LC-MS/MS is the most common method used to quantify polyphenols other methodologies, such as ultra-performance liquid chromatography coupled to quadrupole time of flight (UPLC-QTOF)/MS and quantitative nuclear magnetic resonance (qNMR) spectroscopy offer advantages allowing the quantification of a wide range of metabolites without the need of sample hydrolysis by glucoronidase/sulphatase enzymes prior to analysis, which could compromise the stability of polyphenols that are to be quantified (Ding, *et al.*, 2013; Cheynier *et al.*, 2015).

2.5.4 Conclusion

The use of FFQ and 4-DFD appear to be effective approaches as proxy estimates of compliance to advice to follow polyphenol-rich diet over prolonged periods (FFQ) or during shorter pre-specified periods, although differences in the level of detail of the data collected meant that there was not complete agreement between the two methods. Future research in this area could include the development of a specific FFQ for estimation of polyphenols intake including all classes and main subclasses as was done for 4-DFD in this study. The development, validation and use of a standardised FFQ and updated polyphenol databases in different studies would permit better intake estimation and would make comparisons between populations an achievable goal. The

practicality and validity of this approach will permit use in larger populations such as larger prospective cohort studies. The 4-DFD proved to be an accurate approach for estimate of short-term polyphenol intake as demonstrated by associations with urine polyphenols detected by a more sensitive LC-MS/MS method. However, serious consideration must be taken for the use of those two methods in a large scale study, since its burden and cost might be excessive where a simple indicator of compliance to dietary advice is required.

Healthy (free of overt CVD, T2D and other clinically significant chronic diseases) and probably health-conscious volunteers participated in the CRESSIDA study. Further changes in dietary patterns and other beneficial health effects may have been achieved in a less health-conscious population adhering to UK dietary guidelines. Nevertheless following dietary guidelines did indeed lead to increases in specific polyphenols that have been associated with health benefits (e.g. anthocyanins, isoflavones and lignans), despite the fact that the majority of polyphenol intakes were supplied by tea, coffee, wine and chocolate in this UK population. This suggests that consuming at least 5 portions F&V per day and >50 % of cereal intake as wholegrain will lead to a significant enrichment in polyphenol intakes above and beyond the large amounts of polyphenols supplied by hot beverages, wine and chocolate. This may not have been the case if participants had chosen predominantly F&V, which are low in polyphenols (Chong *et al.*, 2012). In particular estimated anthocyanidin intakes (not easily detected in urine) were increased in both dietary estimation methods, and these have been associated with reduced risk of T2D (Jennings *et al.*, 2014), and acute randomised controlled trials have shown that anthocyanin-rich berry consumption reduces glycaemia (Törrönen *et al.*, 2010, Törrönen *et al.*, 2012c, Törrönen *et al.*, 2012a). However, what component of the berries is responsible is not entirely clear.

Consideration in establishing dietary reference intake (DRI) for polyphenols is a growing interest, although there is still debate in how the process should be managed, models suggests an approach to reach optimal intake values by recommendation of polyphenol-rich F&V (5-a-day) (Williamson and Holst, 2008) and establish values to improve health or prevent disease risk in different life stages (Lupton *et al.*, 2014). Although many considerations must be taken into account (as stability, bioavailability, absorption, etc.) our data suggest that an additional specific guideline, indicating high polyphenol subclass-rich foods may be worth consideration since some polyphenols that

have been associated with health benefits increased there were others associated with cardiovascular protection that did not show an increased intake, such as flavan-3-ols estimated by 4-DFD. A specific recommendation would be useful for the food industry as well; targeting affordable functional foods that supply polyphenols that are less accessible to specific populations. However, further research is required to provide robust interventional evidence for any refinement to current "5-a-day" guidelines, since the relative health impact of other non-nutrient bioactives and fibre contained in low-polyphenol fruits and vegetables is only partially understood. Furthermore, in the light of the fact that NDNS survey data suggest that the UK population are not meeting current recommendations then it would be futile, and possibly counter-productive, to add further complexity to existing public health advice.

Chapter 3

Acute effects of anthocyanin-rich blackcurrant extract and polyphenol-rich apple extracts on postprandial glucose, insulin and gut hormones concentrations and vascular function in healthy men and women. The GLU-BERRY, GLU-APP and GLU-FRU studies.

3.1 Introduction

According to the World Health Organization (WHO), 9 % of the world's population suffers from type 2 diabetes (T2D) and the number is predicted to reach exceptional levels (WHO, 2016). Regular exposure to diets with a high glycaemic load (a measure of the overall blood glucose-raising effect of a serving of a food) and containing high amounts of free sugars may contribute to postprandial hyperglycaemia, a risk factor for T2D (Schulze, 2004, Sluijs *et al.*, 2010, de Koning *et al.*, 2011). Strategies to control chronic postprandial hyperglycaemia by optimising the functionality of foods would strengthen efforts to reduce the risk for T2D. Fruit polyphenols may help to delay glucose absorption and thereby control glucose levels following a carbohydrate-containing meal or beverage. *In vitro* studies suggest that some berry anthocyanins and apple polyphenols are effective inhibitors of digestive enzymes, α-amylases and α-glucosidases (Adisakwattana *et al.*, 2004, Tadera *et al.*, 2006, Adisakwattana *et al.*, 2009, Akkarachiyasit *et al.*, 2010, Pereira *et al.*, 2011). Additional *in vitro* experiments have shown inhibition of intestinal glucose transporters SGLT1 and GLUT2 by berry anthocyanins and apple polyphenols (Johnston *et al.*, 2005, Kwon *et al.*, 2007, Manzano and Williamson, 2010, Alzaid *et al.*, 2013, Schulze *et al.*, 2014). Human data is limited; however randomised controlled trials (RCTs) have supported the *in vitro* results of berry anthocyanins and apple polyphenols lowering postprandial plasma glucose concentrations. Administration of different apple products (Johnston *et al.*, 2002, Schulze *et al.*, 2014, Makarova *et al.*, 2015) and a mixture of whole strawberries, bilberries, lingonberries, chokeberries and blackcurrants (Törrönen *et al.*, 2010, Törrönen *et al.*, 2012a, Törrönen *et al.*, 2012c, Törrönen *et al.*, 2013) have been shown to significantly reduce postprandial glucose concentrations following the consumption of either starch, glucose or sucrose loads.

However, the majority of study designs have varied in their route of administration of polyphenols, making difficult to identify the relative effects of the polyphenols *per se*. Confounding factors from the degree of mastication to variability in the food matrix could influence the release of polyphenols. In addition, the effect of whole fruits or purées used in RCTs could be confounded by the presence of other components as fibre which is known to delay gastric emptying (Jenkins et al., 1978) and thus could be responsible for altering plasma glucose concentrations rather than the polyphenols themselves.

In the present series of RCTs, drinks containing a highly purified anthocyanin-rich blackcurrant extract, highly purified polyphenol-rich apple extract or the combinations of both were tested in reducing the postprandial glycaemia following a carbohydrate-rich meal in a dose-dependent manner, compared with a matched placebo drink. Since RCT of anthocyanin-rich whole foods and individual apple flavonoids have been shown to influence markers of oxidative stress and endothelial function (Egert *et al.*, 2009, Rodriguez-Mateos *et al.*, 2013, Khan *et al.*, 2014, Alvarez-Suarez *et al.*, 2014, Gasper *et al.*, 2014), markers of these were included as additional secondary outcome variables.

3.2 Aim and objectives

Aim

To investigate the extent to which plasma glucose concentrations are reduced following different doses of polyphenol-rich fruit extracts derived from blackcurrants and apples added to a low sugar beverage when consumed with a high carbohydrate test meal.

Objectives

Three randomised controlled crossover trials:

1) the first two to investigate the effects of consumption of different doses of a anthocyanin-rich blackcurrant extract and a polyphenol-rich apple extract incorporated into a low sugar drink on postprandial metabolic responses following a high-carbohydrate test meal;
2) the third to investigate the additive/synergistic effects of consumption of different doses of a polyphenol-rich apple extract and a single dose of an anthocyanin-rich blackcurrant extract incorporated into a low sugar drink on postprandial metabolic responses following a mixed carbohydrate (sugar and starch) test meal;

Primary outcomes

Investigate whether there are dose-related differences in peak postprandial glucose following consumption of a small volume beverage containing either a blackcurrant or apple polyphenol extract together with a high-carbohydrate test meal, compared to a placebo drink.

Secondary outcomes

Investigate differences in postprandial plasma glucose area under the curve, insulin, C-peptide, glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide-1 (GLP-1), triacylglycerol (TAG), non-esterified fatty acids (NEFA) and 8-isoprostane-$F_{2\alpha}$ concentrations and vascular function in order to elucidate potential mechanisms for any differences observed in plasma glucose response.

3.3 Hypothesis

Hypothesis 1 Acute consumption of an anthocyanin-rich blackcurrant extract and a polyphenol-rich apple extract will reduce peak postprandial glucose concentrations following a high-carbohydrate meal.

Hypothesis 2 Acute consumption of anthocyanin-rich blackcurrant extract and polyphenol-rich apple together will have an additive/synergistic effect on peak postprandial glucose concentrations following a high-carbohydrate meal.

3.4 Methodology

The first study consisted of two phases: Phase 1 took 7 months to run, from August 2012 to February 2013, Phase 2 took 6 months to run, from April to September 2013. The study was sponsored by GlaxoSmithKline Nutritional Healthcare (GSKNH) and received approval from the Biomedical Sciences, Dentistry, Medicine and Natural and Mathematical Sciences Research Ethics Subcommittee from King's College London (REC reference: **BDM/11/12-88).** This study abided by the principles outlined in the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013) and was registered at clinicaltrials.gov as NCT01706653.
The second study took 7 months to run, from January to July 2015 and was sponsored by King's College London and received approval from the Biomedical Sciences, Dentistry, Medicine and Natural and Mathematical Sciences Research Ethics Subcommittee from King's College London (REC reference: **BDM/14/15-10).** This study abided by the principles outlined in latest version of the Declaration of Helsinki and was registered at clinicaltrials.gov as NCT02340039.

The lead researcher was Dr Wendy Hall and study investigators included research assistant Ms Leanne Smith, Mr Robert Gray, BSc Nutrition student Najmeh Kamali, BSc Nutrition medical students Stephanie G Stone and Jonathan E Mok, MSc Nutrition students Rhia K Mhajan, Chi Ieng Fu and Georgia Lenihan-Geels. The author's participation in the first study (two phases) comprised assistance in screening and study days as well as blood sample handling and storage following the protocol established by GSK NH and laboratory analysis of 8-isoprostane-$F_{2\alpha}$ concentrations by gas chromatography mass spectrometry (GC-MS). In the second study the author's participation consisted in the preparation of the application for ethical approval and leading and coordinating the trial.

3.4.1 Study design

First study

Phase 1 (**GLU-BERRY study**): A randomised, controlled, double-blind, cross over design was used to allow subjects receive each of the following interventions in random order at 4 separate study visits: 0, 150, 300 and 600 mg of blackcurrant anthocyanins delivered in a blackcurrant extract dispersed in a low sugar fruit drink. At least 7 days wash-out period were required between study days. After baseline measurements and blood samples were taken the test drink and meal were consumed within 5 min, total carbohydrate provided including the drink was 67 g. Drink was consumed within 2 minutes and immediately following consumption of the drink 100 g of white bread (Hovis, London, UK) with 32 g of a low polyphenol apricot jam (Hartley's, Hain Daniels Group, Leeds, UK), providing 1127 kJ and 62 g carbohydrate; 39 g starch and 23 g as sugars, were consumed. Further blood samples were collected at 10 min intervals for the first 30 min and then quarter-hourly until t 90 min and at t 120 min (**Figure 3.1**).

Phase 2 (**GLU-APP study**): A randomised, controlled, double-blind, cross over design was used to allow subjects receive each of the following interventions in random order at 3 separate study visits: 0, 300 and 600 mg of apple polyphenols delivered in an apple extract dispersed in a low sugar fruit drink. At least 7 days wash-out period were required between study days. After baseline measurements and blood samples were taken the test drink and meal were consumed within 5 minutes. Immediately after consumption of the test drink, a mixture of 250 ml water and 82.5 g of dextrose monohydrate (OneOn Nutrition, UK), to provide 75 g of dissolved glucose, was consumed. The study procedure thereafter was identical to Phase 1.

Second study

GLU-FRU study: A randomised, controlled, double-blind, cross over design was used to allow subjects receive each of the following interventions in random order at 3 separate study visits: 0 mg of polyphenols, 1200 mg of apple polyphenols and 600 mg apple polyphenols + 600 mg blackcurrant anthocyanins delivered in an apple and a blackcurrant extract dispersed in a low sugar fruit drink. At least 7 days wash-out period were required between study days. After baseline measurements and blood samples were taken the test drink and meal were consumed within 5 min, total carbohydrate

provided including the drink was 75 g. Drink was consumed within 2 minutes and immediately following consumption of the drink 100 g of white bread (Hovis, London, UK) with 30 g of a low polyphenol apricot jam (Hartley's, Hain Daniels Group, Leeds, UK), providing 1284 kJ and 63 g carbohydrate; 41 g starch and 22 g as sugars, were consumed. The study procedure was identical to the first study.

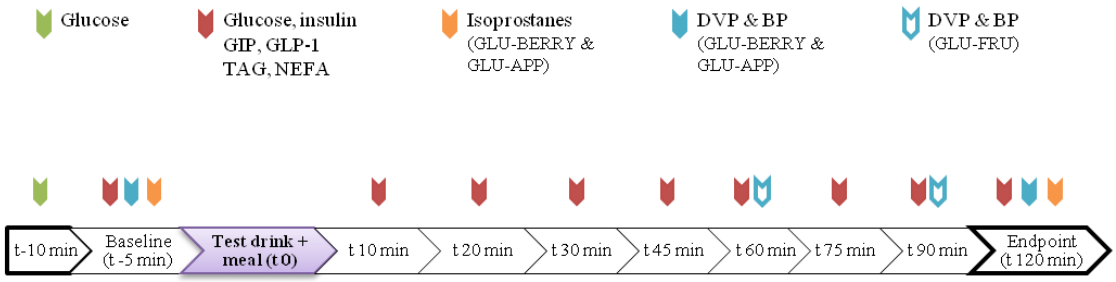


Figure 3.1 Study day timings and procedures

3.4.2 Test drinks and high carbohydrate meals

Highly purified extracts instead of whole fruits were used in test drinks so to avoid the confounding effect of fruit fibre on gastric emptying rate (Ulmius *et al.*, 2009).

GLU-BERRY study

The blackcurrant extract (BerryPharma® by Iprona AG, Lana, Italy) for GLU-BERRY study contained 2822 mg total anthocyanins per 100 ml (5775 mg total polyphenols per 100 ml) and 2.3 g of carbohydrates per 100ml, analysed by RSSL The Lord Zuckerman Research Centre (Reading, UK). Study drinks were formulated by GlaxoSmithKline Nutritional Healthcare R&D (Coleford, UK) and Döhler (Milton Keynes, UK) and standardised to contain 0, 150, 300 or 600 mg anthocyanins. The placebo drink did not have blackcurrant extract added to it, but tannins (polymeric polyphenols, such as proanthocyanidins) were added to lower-concentration drinks and placebo to make sure each of the drinks had comparable astringency/bitterness in taste. Each test drink consisted of a "no added sugar" fruit drink providing 90 kJ, <0.1 g protein, <5 g carbohydrate, and 0.4 g fat, see **Table 3.1.**

Table 3.1 Energy, nutrient and polyphenol composition of the test drinks for GLU-BERRY study

Per 200 ml	**600 mg ACN**	**300 mg ACN**	**150 mg ACN**	**0 mg ACN**
Energy and nutrients				
Energy (kJ)	92	90	90	95
Carbohydrate (g)	4.6	4.5	4.5	4.8
Glucose (g)	0.2	0.2	0.2	0.2
Fructose (g)	0.2	0.2	0.2	0.2
Sucrose/Lactose/Maltose (g)	<0.1	<0.1	<0.1	<0.1
Starch (g)	4.0	4.0	5.0	4.0
Fat *(g)*	0.4	0.4	0.4	0.4
Protein *(g)*	<0.1	<0.1	<0.1	<0.1
Total dietary fibre (AOAC) *(g)*	<0.5	<0.5	<0.5	<0.5
Vitamin C (mg)	<0.5	<0.5	<0.5	<0.5
Vitamin E (mg)	<0.2	<0.2	<0.2	<0.2
Sodium (mg)	<3	<3	<3	<3
Potassium (mg)	15.7	7.8	3.9	<3
Magnesium (mg)	2.3	1.1	0.6	<0.4
Calcium (mg)	6.5	3.3	<3	<3
Phosphorus (mg)	<2	<2	<2	<2
Zinc (mg)	<0.1	<0.1	<0.1	<0.3
Polyphenols				
Total phenolics (mg)[1,2]	1596	810	460	207
Total anthocyanins (mg)[1]	599	322	131	46
Delphinidin-3-rutinoside (mg)[3]	260	140	57	20
Cyanidin-3-rutinoside (mg)[3]	209	113	46	16
Delphinidin-3-glucoside (mg)[3]	76	41	17	6
Cyanidin-3-glucoside (mg)[3]	33	18	7	3

ACN; anthocyanins

[1] Total phenolic and anthocyanin content estimated from direct analysis of drinks by Folin-Ciocalteau method and HPLC respectively.

[2] Tannins added as an ingredient (600 mg ACN 0 mg, 300 mg ACN 60 mg, 150 mg ACN 90 mg, 0 mg ACN 150 mg per 200 ml drink) to render drinks equivalent in bitter/astringent taste for blinding purposes.

[3] Estimated from HPLC analysis of raw extract, not direct analysis of the drinks.

GLU-APP study

The apple extract for GLU-APP (JF-NATURAL, Tianjin Jianfeng Natural Product R&D Co., Ltd, China) contained 73 g of total polyphenols per 100 g of extract, analysed by RSSL The Lord Zuckerman Research Centre (Reading, UK) (**Appendix 1**). The apple extract was ~7% phlorizin and ~63% flavan-3-ols. For GLU-APP study drinks were formulated by GlaxoSmithKline Nutritional Healthcare R&D (Coleford, UK) and standardised to contain 0, 300 or 600 mg of apple polyphenols. Analysis of drinks is pending from Lucozade Ribena Suntory previously GSK.

GLU-FRU study

The blackcurrant extract (BerryPharma® by Iprona AG, Lana, Italy) contained 1.8% anthocyanins (3.6% total polyphenols). The apple extract (Appl'In^{TM} by DIANA FOOD, Antrain, France) contained 67% total polyphenols as analysed DIANA FOOD and confirmed by author using Folin-Ciocalteu method (Singleton and Rossi, 1965). Of total polyphenols 40% were flavonoids (40% flavan-3-ols, 36% dihydrochalcones, 13% flavonols and 11% hydroxycinnamic acids) and extract contained 8.5 g of carbohydrates per 100 g as reported by Diana FOOD (personal communication, confidential information). For GLU-FRU study drinks were formulated by the author and BSc students and standardised to contain 0 mg polyphenols, 1200 mg apple polyphenols and 600 mg apple polyphenols + 600 mg blackcurrant anthocyanins. The extracts were dispersed in a very low-polyphenol double concentrate apple and blackcurrant squash (Robinson's, United Kingdom) made up with water; drinks were matched in macronutrient and energy content. Each test drink consisted of a fruit drink providing 220 kJ, 0.2 g protein and 12 g carbohydrate, see **Table 3.2.** Test drinks (200 ml) were blinded in identical black bottles by an external research technician.

Mixed carbohydrate meal

The standardised high carbohydrate meal consumed on each study visit was freshly prepared on study days previous to baseline measurements, and consisted of 100 g thick sliced white bread (Hovis, London, UK) and Hartley's smooth apricot jam (Hain Daniels Group, Leeds, UK), both high carbohydrate foods are low in polyphenols according to Phenol-Explorer (<6 mg /100 g fresh weight) (Rothwell *et al.*, 2013).

Table 3.2 Energy, nutrient and polyphenol composition of the test drinks for GLU-FRU study

Per 200 ml	**AE**	**AE + BE**	**CON**
Energy and nutrients			
Energy (kJ)	222	227	219
Carbohydrate (g)	12.2	12.2	12.1
Starch (g)	-	-	-
Sugars (g)	12.2	12.2	12.1
of which sucrose (g)	6	6	6
Fat (g)	-	-	-
Protein (g)	0.1	0.2	-
Fibre (g)	0.1	0.4	-
Soluble fibre (g)	0.1	-	-
Polyphenols			
Total phenolics (mg) [1]	1200	1800	4
Total flavonoids (mg) [1]	480	840	2
Flavan-3-ols (mg)[1]	192	96	-
Flavonols (mg) [1]	62.4	31.2	-
Quercetin (mg)[1]	15.6	7.8	
Dihydrochalcones (mg)[1]	172.8	86.4	0.4
Phlorizin (mg)[1]	100	50	-
Hydroxycinnamic acids (mg)[1]	52.8	26.4	1.6
Chlorogenic acids (mg)[1]	31.7	15.9	-
Anthocyanins (mg)[1]	-	600	-
Other polyphenols (mg)[1]	720	960	-

CHO; carbohydrate, AE; 1200 mg of apple polyphenols, AE+BE; 600 mg of blackcurrant anthocyanins + 600 mg apple polyphenols, CON; 0 mg of blackcurrant anthocyanins and apple polyphenols.
[1] Estimated from analysis of raw extract, not direct analysis of the drinks

3.4.3 Sample Size

Biostatistics department GSKNH determined the sample size of the first study, it was established that with 18 subjects, it would be possible to detect a 1.6 mmol/L difference in glucose incremental Cmax, assuming 80% power, a standard deviation of differences of 1.878, estimated from a similar study (Törrönen *et al.*, 2012a) using a paired t-test with a 0.017 (Bonferoni adjustment allowing for 3 treatment comparisons). This was later revised to a sample size of 22 for optimal statistical power.

For the second study, power calculations were done from plasma glucose concentrations obtained from GLU-BERRY study. A sample size of 26 subjects has 80% power to detect a difference between average mean area over baseline values 0-30 min of 0.35 mmol/L·h between test drinks with a significance level of α=0.01 (two-tailed), calculated from a 0.44 mmol/L standard deviation of the difference.

3.4.4 Subjects

Different strategies were used for advertisement of the clinical trials; circular emails within King's College London (KCL), social networking website, poster advertising at KCL, fitness centres and community centres (**Appendices 2, 3, 4**), online advertising agency and nutrition focus groups at community centres. A participant information sheet (**Appendix 5**) or a pdf file was provided to volunteers who expressed interest. Respondents were initially interviewed over the telephone and if eligible (**Appendix 6**), were invited to a screening visit in the Metabolic Research Unit at the Diabetes & Nutritional Sciences Division, King's College London in a fasting state. Screening visit consisted of the measurement of height, weight, waist circumference, % body fat (Tanita^{TM} Body Composition Analyser), supine blood pressure and digital volume pulse. Blood samples were taken, by a trained phlebotomist, for liver function tests, haematology, plasma glucose and lipid profile, written informed consent was given by participants (**Appendix 7**) and a small remuneration was given for participation in the studies. Blood samples were analysed in the Clinical Biochemistry department, King's College Hospital, United Kingdom by Tracy Dew and results were received within one week. A unique screening number identify each subject screened for study participation. Screening numbers were assigned in ascending numerical order as each subject signed their consent form and keep it as the study ID if participant complied with inclusion criteria and did not report any of the exclusion criteria.

3.4.4.1 Inclusion criteria were:

- Male and post-menopausal female aged 20-60 y
- Healthy (free of diagnosed diseases in the exclusion criteria)
- BMI 18-35 kg/m^2
- Able to understand the information sheet and willing to comply with study protocol
- Able to give informed written consent

3.4.4.2 Exclusion criteria were:

- Diagnosed with phenylketonuria,
- Known or suspected food intolerances, allergies or hypersensitivity
- Women who are known to be pregnant or who are intending to become pregnant over the course of the study
- Women who are breast feeding
- Participation in another clinical trial
- Donated blood within 3 months of the screening visit and participants for whom participation in this study would result in having donated more than 1500 millilitres of blood in the previous 12 months.
- Full blood counts and liver function test results outside of the normal range.
- Pre-menopausal women due to the potential influence of cyclical changes in reproductive hormones on insulin sensitivity
- Current smokers, or reported giving up smoking within the last 6 months
- History of substance abuse or alcoholism
- Reported history of CVD, diabetes (or fasting glucose ≥ 7.1 mmol/L), cancer, kidney, liver or bowel disease, gastrointestinal disorder or use of drug likely to alter gastrointestinal function
- Unwilling to restrict consumption of specified high polyphenol foods for 24 h before the study
- Weight loss >3 kg in preceding 2 months and body mass index <18 or >35 kg/m^2
- Blood pressure ≥160/100 mmHg
- Total cholesterol ≥ 7.5 mmol/L; fasting triacylglycerol concentrations ≥ 5.0 mmol/L

- Medications that may interfere with the study such as alpha-glucosidase inhibitors (acarbose: Glucobay), insulin-sensitising drugs (metformin: Glucophage, Glucophage SR, Eucreas, Janume; thiazolidinediones: Actos, Competact), sulfonylureas (Daonil, Diamicron MR, Glibenese, Minodiab, Amaryl Tolbutamide), and lipid-lowering drugs (statins, nicotinic acid, colestyramine anhydrous, ezetimibe, fibrates). Other medications should be reviewed by medical representative from KCL on a case by case basis.
- Nutritional supplements that may interfere with the study such as higher dose vitamins/minerals (>200 % reference nutrient intake (RNI)), B vitamins, vitamin C, calcium, copper, chromium, iodine, iron, magnesium, manganese, phosphorus, potassium and zinc. Subjects already taking vitamin or minerals at a dose around 100 % or less up to 200 % of the RNI, or evening primrose/algal/fish oil supplements will be asked to maintain habitual intake patterns, ensuring that they take them every day and not sporadically. They will be advised not to stop taking supplements or start taking new supplements during the course of the study.

3.4.5 Randomisation procedure

The lead investigators reviewed all criteria and signed the case report form to confirm the subject's eligibility to participate in the study. Subjects who meet all inclusion and exclusion criteria were randomised according to the randomisation schedule. Randomisation numbers were assigned in ascending numerical order as each subject was determined to be fully eligible. The randomisation schedule for GLU-BERRY and GLU-APP studies were provided by the Biostatistics Department, GSKNH and the randomisation schedule for GLU-FRU study was created using Research Randomizer software (https://www.randomizer.org). The allocation of treatment was blinded from the investigators, laboratory technicians, statisticians and the study participants by independent contracted parties within GlaxoSmithKline Nutritional Healthcare at GLU-BERRY and GLU-APP studies and by external research technician at GLU-FRU study. The blinding could only been broken in an emergency where it was essential to know which treatment a subject received in order to give the appropriate medical care. Randomisation number was in the format starting FPA001 for GLU-BERRY and GLU-APP studies and GFS001 for GLU-FRU study. The format of randomisation number was used to labelling tubes, vials and cryovials used along studies (**Table 3.3**). All randomised subjects were recorded on the participant identification log.

Table 3.3 Randomisation number and labelling format of cryovials

STUDY NAME	SUBJECT	VISIT	TIME POINT
FPA/GFS	001	1/v1	0
VACUTAINER/TEST			
Ser/NEFA			
DATE			
20/09/2012			

3.4.6 Study day

On the day previous to each study visit, participants were told not to participate in strenuous exercise and to avoid alcohol, caffeine, oily fish, high polyphenol foods (from a list provided) and foods high in fat. Participants were asked to consume a low-fat meal the evening before each visit and to fast 12 h before, avoid eating or drinking anything except for water until the morning of the study visit. Detailed dietary and lifestyle guidance was provided to each participant before commencing the study period. Participants attended the Metabolic Research Unit at King's College London between 08.00 and 11.00 h. On arrival to study days, participants reported any deviations from the protocol, if any deviation they were weighed and rested in the supine position for ten minutes. Digital volume pulse (DVP) and blood pressure (BP) measurements were recorded for triplicate. For blood sampling a venous cannula was inserted in the forearm vein by a trained phlebotomist and baseline samples for analysis of glucose, insulin, triacylglycerol (TAG), non-esterified fatty acids (NEFA), glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like-peptide-1 (GLP-1) were collected. Glucose baseline samples were taken in duplicate (at -10 and -5 min) and isoprostanes samples were taken at baseline and endpoint min for GLU-BERRY and GLU-APP studies. After baseline measurements and blood sampling participants consumed the drink immediately before a high-carbohydrate meal. Following the consumption of the test meal, blood samples were taken at minutes 10, 20, 30, 45, 60, 75, 90 and 120. BP and DVP measurements were taken again at min 120 and two more time points were added for GLU-FRU study at 60 and 90 min. See **Fig 3.1** for study day timings and procedures.

3.4.7 Methods

3.4.7.1 Dietary intake analysis

For GLU-FRU study participants were asked to complete a 4-day food diary (4-DFD) previous to the first visit day. Baseline macronutrient intake was assessed using Nutritics software (Nutritics, version 3.5. Northern Ireland) and polyphenol intake was assessed using the database created for the polyphenol intake analysis of 4-DFD of participants in the CRESSIDA study (Chapter 2).

3.4.7.2 Blood pressure

Blood pressure was measured at the brachial artery using a calibrated automated blood pressure monitor (Omron 705IT, Omron Healthcare Europe B.V.). Participants were allowed to rest quietly, in supine position, at a comfortable room temperature for ten minutes before the measurements were performed. Participants were not drinking, eating or talking at the time that the blood pressure was measured. The arm that the measurement was taken from was comfortably supported to heart level. The cuff was place and the bladder of the cuff was 2-3 cm above the antecubital fossa in the brachial artery (around the participants' upper arm). The cuff was wrapped snugly around the arm, making sure that the cuff index line fell within the marked ranges allowing space for a finger between the participants' arm and the cuff. The cuff was placed so that the participants' artery was aligned with the cuff arrow marked 'artery'. The start/stop button was pressed allowing the reading to take place. Readings for systolic and diastolic blood pressure were written down in the case report form (CRF), as well as the heart rate readings. Repeated measurements of blood pressure were done two more times at two - five minute intervals, and the first one was discarded.

3.4.7.3 Digital volume pulse measurements

Arterial tone was measured using the digital volume pulse (DVP). The DVP was obtained by photoplethysmography (PulseTrace PCA 2, Micro Medical Ltd, Kent, UK) and used to calculate stiffness index (DVP-SI, m/s) and reflection index (DVP-RI, %). DVP-SI is related to large artery stiffness and correlates closely with large artery pulse wave velocity (PWV) (Millasseau et al. 2002). Although DVP-SI is a measure of arterial stiffness which increases with age, it is also sensitive to small changes in vascular tone induced by vasodilators (for example, glycerol trinitrate), and it has been demonstrated to be sensitive to short-term fluctuations in vessel tone postprandially (Hall et al 2008). DVP-RI is more strongly related to vascular tone of small arteries and

is markedly sensitive to drugs influencing vasomotor tone (Chowienczyk et al., 1999; Millasseau et al., 2002). DVP-RI shows marked changes in response to vasoconstrictors and vasodilators. The DVP was measuring using an infra-red probe that clips on the finger/thumb of any hand (preferably index finger of the non-dependant hand). Participants were allowed to rest quietly, in supine position, at a comfortable room temperature for ten minutes before the measurements were performed. The measurements were done three times, using the equipment default settings, separated by 30-45 seconds. The three values for SI were within 10-15% of each other. If one was clearly irregular was be discarded. Values were registered in the cases report form.

3.4.7.4 Blood sampling

For blood sampling, an 18 or 20 G IV catheter (BD Venflon^{TM} Pro Safety Shielded, Cat no. 393226 (green) or 393224 (pink)) was inserted, ideally, into a vein in the antecubital fossa of the arm. If those veins were not suitable then the cannula was inserted into the cephalic vein of the lower forearm. The catheter was held in place with an IV dressing (BD Veca IV dressing, Cat no. 392020), blood was drawn using a 3-way tap (BD 3-way taps Cat no. 344995). The 2 h blood samples were collected by a trained phlebotomist using 5 or 20 ml syringes according to the blood collection protocol outlined in **Fig 3.1.** A baseline blood sample was taken for the measurement of plasma glucose, insulin, C-peptide, TAG, NEFA, GIP and GLP-1 and isoprostanes. Further blood samples for plasma glucose, insulin, C-peptide, TAG, NEFA, GIP and GLP-1 were taken at 10, 20, 30, 45, 60, 75, 90 min and additional for isoprostanes at 120 min (**Fig 3.1**). Blood samples were collected into fluoride/oxalate tubes (BD Vacutainer® Cat no. 368921) for glucose analysis, into SST^{TM} serum tubes (BD Vacutainer® Cat no. 367954) for insulin, C-peptide, TAG and NEFA analysis, into K_2EDTA tubes for GIP and GLP-1 (BD Vacutainer® Cat no. 367839). Tubes for GLP-1 analysis had 10 µl per ml blood dipeptidyl peptidase IV inhibitor added (Millipore, MO, USA). Blood samples for 8-isoprostane-$F_{2\alpha}$ analysis were collected into chilled citrated tubes (BD Vacutainer Cat no. 367691) and chilled fresh indomethacin (cyclooxygenase inhibitor) was immediately added (final concentration 15 µmol/L). The sample was kept on ice 30 min prior to centrifugation. Prior to centrifugation BHT was added (final concentration 20 µmol/L) and the samples were snap frozen in liquid N_2. Participants were provided with lunch after the last measurement was taken and the study visit ends.

3.4.7.5 Sample analysis

Following collection into vacutainers tubes and prior to centrifugation, samples were placed in ice. After centrifugation at 1300 g , 4 ° C for 15 min samples were aliquot into their correspondent labelled cryovial (**Table 3.3**) and frozen at -40 or -80 ° C for their subsequent analysis (**Table 3.4**).

Table 3.4 Blood sampling protocol for GLU-BERRY and GLU-APP studies

SAMPLE	TUBES	Order draw	ICE/ RT	CENTRIFUGE	SEPARATION	ANALYTES	ANALYSIS	STORAGE
Fasting (-10 min)	4 ml fluoride oxalate	1	ICE	15min X 1300g @ 4^{O}C	1 ml	Glucose	KCL – ILAB	-40 ^{o} C
(4 ml)	(grey)				1 ml	Spare		-40 ^{o} C
Fasting (-5 min)	4 ml fluoride oxalate	1	ICE	15min X 1300g @ 4^{O}C	1 ml	Glucose	KCL – ILAB	-40 ^{o} C
(21 ml)	(grey)				1 ml	Spare		-40 ^{o} C
	5 ml serum	1	ICE	15min X 1300g @ 4^{O}C	0.5 ml	TAG	KCL – LAB	-40 ^{o} C
	(gold)				0.5 ml	Insulin	KCH	-40 ^{o} C
					0.5 ml	NEFA	KCL - LAB	-40 ^{o} C
					0.5 ml	spare		-40 ^{o} C
	4 ml EDTA + DPP IV inhibitor	2	ICE	15min X 1300g @ 4^{O}C	0.5 ml	GIP	KCH	-80 ^{o} C
	(lavender)				0.5 ml	GLP-1	KCH	-80 ^{o} C
					1 ml	spare		-80 ^{o} C
	2 x 4 ml pre-chilled citrate	3	ICE	15min X 1300g @ 4^{O}C	2.2 ml	Isoprostanes	KCL	-80 ^{o} C
	(blue)			(+snap freeze)	2.2 ml	Isoprostanes	KCL	-80 ^{o} C
TEST MEAL (0 min)								
Postprandial (10, 20, 30,	4 ml fluoride oxalate	1	ICE	15min X 1300g @ 4^{O}C	1 ml	Glucose	KCL – ILAB	-40 ^{o} C
45, 60, 75, 90 min)	(grey)				1 ml	Spare		-40 ^{o} C
(13 ml)	4 ml serum	2	ICE	15min X 1300g @ 4^{O}C	0.5 ml	TAG	KCL – ILAB	-40 ^{o} C
	(gold)				0.5 ml	Insulin	KCH	-40 ^{o} C
					0.5 ml	NEFA	KCL – ILAB	-40 ^{o} C
					0.5 ml	spare		-40 ^{o} C
	5 ml EDTA + DPP IV inhibitor	3	ICE	15min X 1300g @ 4^{O}C	0.5 ml	GIP	KCH	-80 ^{o} C
	(lavender)				0.5 ml	GLP-1	KCH	-80 ^{o} C
					1 ml	spare		-80 ^{o} C
Postprandial (120 min)	4 ml fluoride oxalate	1	ICE	15min X 1300g @ 4^{O}C	1 ml	Glucose	KCL – ILAB	-40 ^{o} C
(21 ml)	(grey)				1 ml	Spare		-40 ^{o} C
	4 ml serum	2	ICE	15min X 1300g @ 4^{O}C	0.5 ml	TAG	KCL – ILAB	-40 ^{o} C
	(gold)				0.5 ml	Insulin	KCH	-40 ^{o} C
					0.5 ml	NEFA	KCL – ILAB	-40 ^{o} C
					0.5 ml	spare		-40 ^{o} C
	5 ml EDTA + DPP IV inhibitor	3	ICE	15min X 1300g @ 4^{O}C	0.5 ml	GIP	KCH	-80 ^{o} C
	(lavender)				0.5 ml	GLP-1	KCH	-80 ^{o} C
					1 ml	spare		-80 ^{o} C
	2 x 4 ml pre-chilled citrate	4	ICE	15min X 1300g @ 4^{O}C	2.2 ml	Isoprostanes	KCL	-80 ^{o} C
	(blue)			(+snap freeze)	2.2 ml	Isoprostanes	KCL	-80 ^{o} C

Table 3.4 Blood sampling protocol for GLU-FRU study (continued)

SAMPLE	TUBES	Order draw	ICE/ RT	CENTRIFUGE	SEPARATION	ANALYTES	ANALYSIS	STORAGE
Fasting (-10 min)	4 ml fluoride oxalate	1	ICE	15min X 1300g @ 4^{O}C	1 ml	Glucose	KCL – ILAB	-40 ^{o} C
(4 ml)	(grey)				1 ml	Glucose	KCL– ILAB	-40 ^{o} C
					1 ml	Spare		-40 ^{o} C
Fasting (-5 min)	4 ml fluoride oxalate	1	ICE	15min X 1300g @ 4^{O}C	1 ml	Glucose	KCL – ILAB	-40 ^{o} C
(13 ml)	(grey)				1 ml	Glucose	KCL – ILAB	-40 ^{o} C
					1 ml	Spare		-40 ^{o} C
	5 ml serum (gold)	2	RT(10')	15min X 1300g @ 4^{O}C	0.5 ml	Insulin	KCH	-40 ^{o} C
					0.5 ml	TAG	KCL – ILAB	-40 ^{o} C
					0.5 ml	NEFA	KCL – ILAB	-40 ^{o} C
					0.5 ml	Spare		-40 ^{o} C
					0.5 ml	Spare		-40 ^{o} C
	4 ml EDTA + DPP IV	3	ICE	15min X 1300g @ 4^{O}C	0.5 ml	GIP	KCH	-80 ^{o} C
	inhibitor (lavender)				0.5 ml	GLP-1	KCH	-80 ^{o} C
					1 ml	Spare		-80 ^{o} C
					1 ml	Spare		-80 ^{o} C
				TEST MEAL (0 min)				
Postprandial	4 ml fluoride oxalate	1	ICE	15min X 1300g @ 4^{O}C	1 ml	Glucose	KCL – ILAB	-40 ^{o} C
(10, 20, 30, 45, 60, 75,	(grey)				1 ml	Glucose	KCL – ILAB	-40 ^{o} C
90 and 120 min)					1 ml	Spare		-40 ^{o} C
(13 ml)	5 ml serum (gold)	2	RT(10')	15min X 1300g @ 4^{O}C	0.5 ml	Insulin	KCH	-40 ^{o} C
					0.5 ml	TAG	KCL – ILAB	-40 ^{o} C
					0.5 ml	NEFA	KCL – ILAB	-40 ^{o} C
					0.5 ml	Spare		-40 ^{o} C
					0.5 ml	Spare		-40 ^{o} C
	4 ml EDTA + DPP IV	3	ICE	15min X 1300g @ 4^{O}C	0.5 ml	GIP	KCH	-80 ^{o} C
	inhibitor (lavender)				0.5 ml	GLP-1	KCH	-80 ^{o} C
					1 ml	Spare		-80 ^{o} C
					1 ml	**Spare**		-80 ^{o} C

3.4.7.5.1 Plasma glucose

Plasma glucose concentrations were analysed at KCL on the ILab 650 chemistry analyser. The IL Glucose Oxidase reagent was supplied ready to use (IL^{TM} Test Cat No. 0018259140). Glucose was measured using the enzymes glucose oxidase and peroxidase. In the first step glucose was converted to gluconic acid and hydrogen peroxide. The hydrogen peroxide then reacts, in the presence of peroxidase, 4-aminophenazone and phenol to produce a red quinoneimine dye. The increase in absorbance generated by the red dye was proportional to the glucose concentration in the sample. Primary measurements were taken at 510 nm, and a blanking reading was taken at 600 nm. Precision: the coefficient of variation for two quality control serum samples 7.0-15.2 mmol/L was not be greater than 2% within run and total.

3.4.7.5.2 Insulin

Plasma insulin concentrations were analysed at KCH by immunoassay. Insulin reagent supplied by Siemens Medical Solutions Diagnostics Europe Ltd. The Siemens Advia Centaur assay is a two-site sandwich immunoassay using direct chemiluminometric technology, which uses constant amounts of two antibodies. Sample is incubated with two insulin-specific antibodies. The first is in the Lite reagent, is a monoclonal mouse anti-insulin antibody labelled with acridinium ester. The second antibody, in the solid phase, is a monoclonal mouse anti-insulin antibody, which is covalently coupled to paramagnetic particles. Insulin forms a sandwich between the two antibodies. After the incubation a magnetic field is applied causing the solid phase (including the sandwich) to be held at the site of the reaction cuvette while the liquid phase is aspirated. The cuvette is then washed with deionised water. Acid reagent (containing hydrogen peroxide) is then added to the cuvette to begin the light emission reaction with the acridinium ester. The cuvette is then moved to the luminometer and base reagent is added to enhance the light reaction. Light intensity is measured immediately and converted to relative light units. This has a direct proportional relationship with the insulin concentration. Sensitivity: Minimum detectable concentration of 0.5 mU/L. Assay Range: Serum concentrations up to 300 mU/L.

3.4.7.5.3 C-peptide

Plasma C-peptide concentrations were analysed at KCH by immunoassay. This was a sandwich ELISA based assay. Biotinylated monoclonal and horseradish peroxidase (HRP) labelled antibodies were used; both have high affinity and specificity for different epitopes of C-Peptide. Reaction between the C-Peptide antibodies and native C-Peptide occurred without competition or steric hindrance forming a soluble sandwich complex. After incubation and washing TMB substrate solution was added to be catalysed by HRP and produce blue coloration. The reaction was terminated adding stop solution, which stopped the blue colour development and produced a yellow colour. The intensity of signal was measured at 450 nm and was directly proportional to the amount of C-peptide in the sample. The detection limit was 25 pmol/L.

3.4.7.5.4 Triacylglycerol (TAG)

Plasma TAG concentrations were analysed at KCL on the ILab 650 autoanalyser, utilising an *in vitro* enzymatic colorimetric method (IL^{TM} Triglycerides, Cat No. 0018258740) for the quantification of the red pigment that is produced, which is proportional to the concentration of TAG. The red quinoneimine is produced by a multi-enzymatic cascade reaction, using enzymes; lipase, glycerol kinase, glycerophosphate oxidase and peroxidise to degraded triglycerides to the red pigment. Primary measurements were taken at 510 nm, and a blanking reading was taken at 700 nm. Precision: the coefficient of variation for two quality control serum samples 1.12-3.0 mmol/L, was not be greater than 2 % within run and total.

3.4.7.5.5 Total non-esterified fatty acids (NEFA)

Plasma NEFA concentrations were analysed at KCL on the ILab 650 autoanalyser, utilising an *in vitro* enzymatic colorimetric method (NEFA, Randox Cat. No. FA 115) for the quantification of the purple pigment that is produced, which is proportional to the concentration of NEFA. The purple adduct is produced by a multi-enzymatic cascade reaction, using enzymes; Acyl-CoA synthetase, Acyl-CoA oxidase and peroxidise to degraded NEFA to the purple pigment. Precision: the coefficient of variation for two quality control serum samples 0.58-1.46 mmol/L, was not be greater than 3 % within run and total.

3.4.7.5.6 Plasma GIP and GLP-1

Plasma GIP and GLP-1 concentrations were analysed by immunoassay at the Clinical Biochemistry laboratory, Kings College Hospital by Dr Tracy Drew. The dual-monoclonal, sandwich immunoassay was used for GIP, and the high sensitivity GLP-1 active ELISA kit, chemiluminescent for GLP-1.

Plasma GIP

Linco Research. 6 Research Park Dr St Charles, Missouri 63304 USA. This kit is for non-radioactive quantification of human GIP in human serum, plasma, tissue extract and cell culture samples. This kit has 100 % cross reactivity to human GIP (1-42) and GIP (3-42). This assay is a Sandwich ELISA based, sequentially, on: 1) capture of human GIP molecules from samples to the wells of a microtiter plate coated by a pre-titered amount of anti-GIP monoclonal antibodies, 2) wash away of unbound materials from samples, 3) binding of a second biotinylated anti-GIP polyclonal antibody to the captured molecules, 4) wash away of unbound materials from samples, 5) incubation of streptavidin-horseradish peroxidase conjugate to bind to the immobilized biotinylated antibodies, 6) wash away of free enzyme conjugates, and 7) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5' tetramethylbenzidine. The enzyme activity was measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590 nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured human GIP in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of human GIP. Sensitivity: The minimal detectable GIP concentration was 4.2 pg/ml.

Plasma GLP-1

Linco Research. 6 Research Park Dr St Charles, Missouri 63304 USA. This kit is for non-radioactive quantification of biologically active forms of Glucagon-Like Peptide-1 [i.e. GLP-1 (7-36 amide) and GLP-1 (7-37)] in plasma and other biological media. It is highly specific for the immunologic measurement of active GLP-1 and do not detect other forms of GLP-1 (e.g. 1-36 amide, 1-37, 9-36 amide or 9-37). The GLP-1 sequence is highly conserved between the species, with no sequence variation occurring in all mammals. This assay is based, sequentially, on: 1) capture of active GLP-1 from the

sample by a monoclonal antibody, immobilised in the wells of a microwell plate, that binds specifically to the N-terminal region of active GLP-1 molecule, 2) washing to remove unbound materials, 3) binding of an anti-GLP-alkaline phosphatise detection conjugate to the immobilised GLP-1, 4) washing off unbound conjugate, and 5) quantification of bound detection conjugate by adding MUP (methyl umbelliferyl phosphate) which in the presence of alkaline phosphatise forms the fluorescent product of umbelliferon. Since the amount of fluorescence generated is directly proportional to the concentration of active GLP-1 in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of active GLP-1. Sensitivity; the minimal detectable GLP-1 concentration was 1.5 pM.

3.4.7.5.7 Plasma isoprostanes

Isoprostanes (8-isoprostane $F_{2\alpha}$) were analysed using immunoaffinity gas chromatography negative chemical ionization mass spectrometry (GC-NCI-MS) with prior immunoaffinity purification. In the isoprostanes analysis the research technician Robert Gray advised in the development of the protocol and was in charge of the GC-NCI-MS equipment. Material and reagents are listed in **Table 3.5**; the protocol followed is described below.

Table 3.5 Material, reagents and solutions details for Isoprostanes (8-isoprostane $F_{2\alpha}$) analysis

Material, reagent or solution	Concentration	Supplier details
8-Isoprostane affinity column (4ml)		Cayman Chemical Company, cat. no.400058
Eicosanoid affinity column buffer solution (EACB): 13.3 g K_2HPO_4, 3.22 g KH_2PO_4, 0.5 g NaN_3 and 29.2 g NaCl in a final volume of 1 L and pH of 7.4	0.1 M	
Affinity column elution solution (ES)	95% v/v ethanol-ultrapure water	
8-iso-PGF2α (working standard solution)	10 µg/ml	Santa Cruz Biotechnology, cat. no. sc-203219
8-iso-PGF2α-D4 (internal standard solution)	50 ng/ml	Santa Cruz Biotechnology, cat. no. sc-205468
Potassium hydroxide (KOH)	15 % w/v	
Potassium dihydrogen phosphate (KH_2PO_4)	1 M	
N,N-diisopropylethylamine (DIPEA) in acetone	10 % v/v	Sigma-Aldrich; cat. no. 7887685
Pentafluorobenzyl bromide (PFBBr) in acetone	20 % v/v	Sigma-Aldrich, cat. no. 101175241
N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA)	As supplied	Sigma-Aldrich, cat. no. 33024
Isooctane		Sigma-Aldrich, cat. no. 59045

Determination of total (free and esterified) 8-isoprostane $F_{2\alpha}$ in plasma by GC-NCI-MS.

a) Alkaline hydrolysis protocol

Two millilitres aliquots of plasma sample were subjected to alkaline hydrolysis by adding 1 ml of KOH solution in the presence and 1 ng of 8-iso-$PGF_{2\alpha}$-D4 internal standard, incubating mixture for 1 h at 60 ° C.

b) Purification protocol

Hydrolysate was then neutralised with 3-4 ml of KH_2PO_4 solution to give a final pH of 7-7.4 and then buffered with 2 ml of EACB. To extract the total 8-isoprostane $F_{2\alpha}$ of the plasma sample, the buffer mixture was applied to the 8-isoprostane immunoaffinity column and column was washed with 2 ml of EACB, followed by 2 ml of ultrapure water. Washing was done under gravity alone and the washing product discarded. Total 8-isoprostane $F_{2\alpha}$ was eluted by adding 2 ml of ES into the column. Elute was collected in a 2 ml amber vial (VWR International Chromacol Cat no. CRMA2-SVWAST-CPK) and evaporated to dryness using a vacuum centrifugal concentrator at 60°C (Eppendorf concentrator 5301).

c) Regeneration of columns

Columns were used 3 times, after each purification protocol columns were regenerated by washing them with 5 ml of ultrapure water followed by 5 ml of EACB. The columns were store at 4°C with 2 ml of EACB to prevent column packing material to becoming dry.

d) Derivatisation protocol

To the dried sample obtain from the purification protocol was added 25 µL of DIPEA and 25 µL of PFBBr, gently vortex, incubated for 10 min at 60°C and evaporated to dryness under a stream of nitrogen at room temperature. The pentafluoryl benzoyl derivatives formed were then supplemented with 25 µL of BSTFA and 5 µL of DIPEA, gently vortex, incubated for 3 min at 60°C and evaporated to dryness under a nitrogen stream at room temperature. To the trimethyl silyl derivatives formed in these step were added 20 µL of isooctane, vortex and transferred to a GC vial amber fixed insert (Essex Scientific Laboratory Supplies Ttd. Cat no. 03-FISV-A) for GC-MS analysis.

e) Preparation of calibration standards

Serial dilutions of the 8-iso-PGF_{2a} working standard solution, adding 1 ng of internal standard solution (8-iso-$PGF_{2\alpha}$-D4) were prepared for plotting the calibration curve. Concentration points were; 0 ng (blank), 0.05 ng, 0.1 ng and 1.0 ng. Standards solutions were evaporated to dryness at 60°C using a vacuum centrifugal concentrator and derivatised in accordance with the derivatisation protocol.

f) GC-NCI-MS analysis.

The trimethyl silyl derivatives of standards and samples were analysed by GC-MS in negative chemical ionization mode with methane as reagent gas on an Agilent Technologies 6890N/5673 gas chromatograph mass spectrometer. Each standard or sample produces the m/z 569 ion current chromatogram (8-iso-$PGF_{2\alpha}$) and the m/z 573 ion current chromatogram (8-iso-$PGF_{2\alpha}$-D4).

g) Calculations

A calibration curve was constructed of the ratio of peak area of 8-iso-$PGF_{2\alpha}$ to that of the internal standard in the standards against concentration of 8-iso-$PGF_{2\alpha}$ in the standards. 8-iso-$PGF_{2\alpha}$ concentration in the samples was calculated by using the peak area ratios (8-iso-$PGF_{2\alpha}$ to internal standard) in the linear regression equation generated from the standard calibration curve.

3.4.8 Statistical analysis

GLU-BERRY and GLU-APP studies: GSK statisticians were responsible for the data management and final statistical analysis. Data was analysed by analysis of variance with covariates. The model included factors for subject (a random effect), period, treatment and two baseline terms as covariates; (i) the subject-level baseline-number of valid responses calculated as the mean baseline across all periods within a subject, and (ii) the period level baseline (glucose or insulin concentration) minus the subject-level baseline. The residual variance from the model was used to construct confidence intervals for the difference between test treatment and the reference treatment.

GLU-BERRY study:

A linear mixed effects model was used to analyse incremental Cmax and area over baseline (AOB) using PROC MIXED in SAS software (Marlow, UK). AOB was calculated as the positive incremental area or the AUC above baseline (Allison *et al.*, 1995). Main effects of treatment and treatment x time interactions for the change from baseline at each time point were calculated by linear mixed effects modelling using SPSS Statistics version 21 (IBM, UK). The models included subject as a factor (a random effect), fixed factors were treatment (and time and treatment x time interaction where appropriate) and period. Baseline values, and two baseline terms were included as covariates: (i) subject-level baseline; the number of valid responses calculated as the mean baseline across all periods within a subject, and (ii) the period-level baseline minus the subject-level baseline. *P*-values were adjusted using Dunnett's procedure for the comparison against a control (reference) product, either using SAS for incremental Cmax, Tmax and AOB, or using 2-way repeated measures ANOVA in GraphPad Prism version 6.00 for Windows (GraphPad software, CA, USA) for treatment and treatment x time effects (since SPSS does not provide Dunnett's multiple testing adjustment in the linear mixed model facility with repeated measures). The assumption of normality and homogeneity of variance was investigated. Violation of these assumptions were overcome where appropriate using natural logarithmic transformation or performing a non-parametric test. Tmax data was analysed by Friedman's non-parametric test using GraphPad Prism.

GLU-FRU study: Area under the curve (AUC) and incremental area under the curve (iAUC) were calculated using the trapezoidal rule. iAUC was calculated by subtracting baseline values from all subsequent time-point values (Allison *et al.*, 1995). Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) v.21. Normality of data distribution was evaluated by inspection of histograms and normal Q-Q plots. Non-normally distributed data was natural logarithmic converted and analysed. Two-way repeated measures analysis of variance (ANOVA) were used for treatment and treatment x time interactions, iAUC 0-120 min, iAUC 0-30 min and Cmax. A non-parametric Friedman's test was used to detect a significant difference in Tmax between the treatments. *P*-values were adjusted using two-way repeated measures ANOVA with post hoc analysis by Tukey's adjustment for treatment and treatment x time interactions (GraphPad Prism).

3.5 Results

3.5.1 GLU-BERRY study

A total of 30 healthy men and postmenopausal women aged 20 to 60 years attended screening sessions, 26 meet all inclusion criteria and therefore were found eligible to participate in the study. Participants were randomised to treatment and 22 subjects completed the GLU-BERRY study. Details of study stages are shown on the consort diagram (**Figure 3.2**) and characteristics of the 22 participants who completed the study are shown in **Table 3.6**.

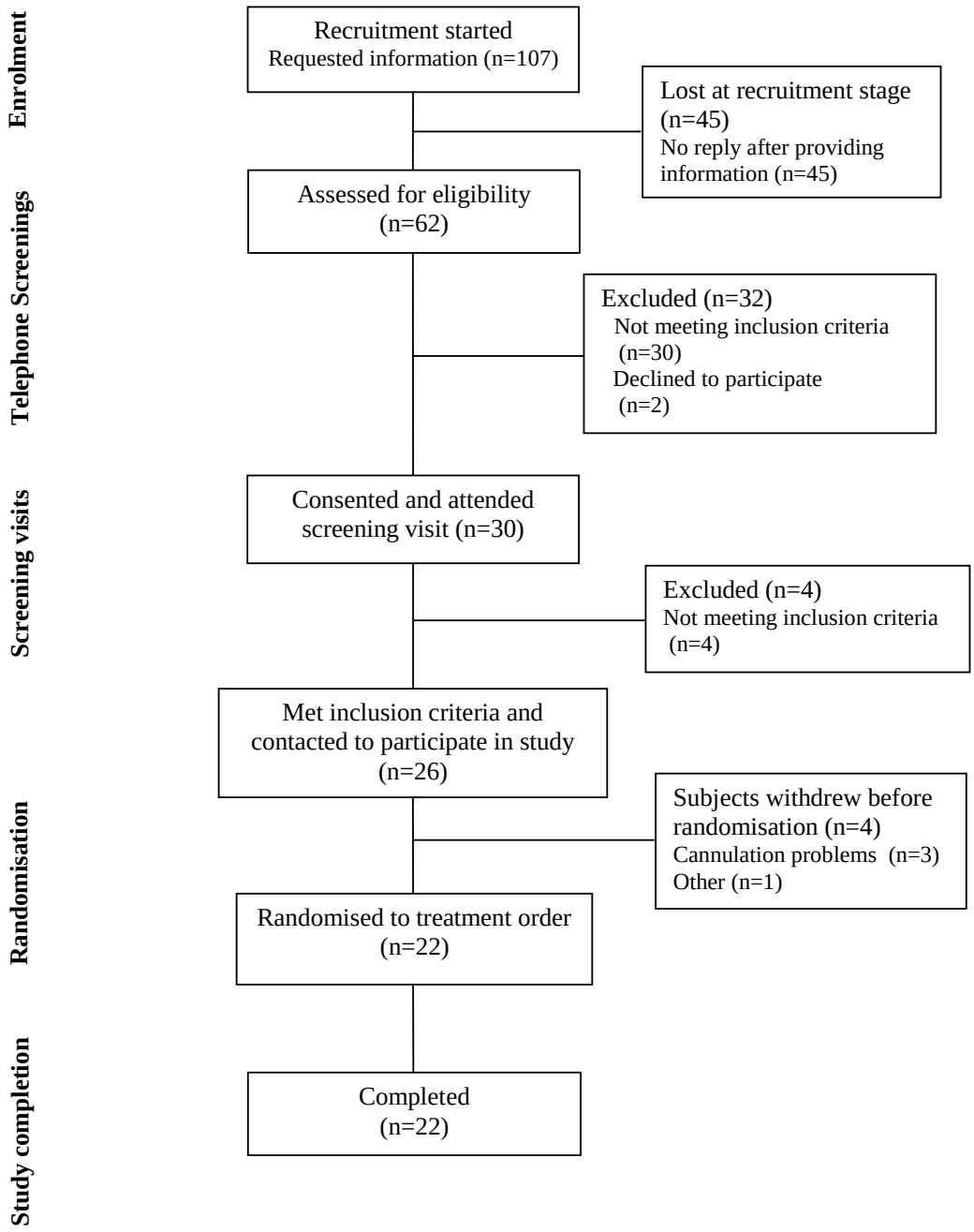


Figure 3.2 Consort diagram for GLU-BERRY study

Table 3.6 Characteristics of GLU-BERRY study population (n=22)

Variable [1]	
Age (y)	45.4 (13.7)
Sex (male to female ratio)	13:9
Body Mass Index (kg/m^2)	25.5 (3.8)
Systolic blood pressure (mmHg)	122.7 (14.1)
Diastolic blood pressure (mmHg)	77.2 (10.8)
Waist circumference (cm)	
Males	88.3 (9.6)
Females	89.2 (13.9)
Body fat (%)	
Males	18.1 (6.2)
Females	35.1 (8.2)
Fasting plasma glucose (mmol/L)	5.4 (0.5)
Fasting plasma triacylglycerol (mmol/L)	1.2 (0.5)
Fasting plasma total cholesterol (mmol/L)	4.9 (1.0)
Fasting plasma LDL cholesterol (mmol/L)	2.8 (0.8)
Fasting plasma HDL cholesterol (mmol/L)	1.5 (0.4)
Males	1.3 (0.4)
Females	1.7 (0.3)

[1]Values are means (SD)

There was an overall treatment effect on change from baseline of (*Ln*)glucose concentrations 0-120 min (P <0.05), with no statistically significant treatment x time interaction for postprandial glucose response (P=0.16) (**Figure 3.3**). Following 600 mg ACN treatment glucose concentrations were inhibited during the initial 30 min of the postprandial period, see **Table 3.7** for details; AOB 0-120 min were not significantly different between treatments. Post hoc pairwise comparisons showed there were significantly lower glucose concentrations following 600 mg ACN compared to 0 mg ACN at 10-30 min post-drink, and there was a statistically significant increase in glucose following 600 mg ACN at 75 min relative to 0 mg ACN (mean difference in change from baseline values was 0.72 mmol/L (0.18, 1.25; P <0.01). Cmax (adjusted for baseline) was not significantly different between treatments. Tmax was slightly greater following 600 mg ACN (mean at 55 min, 95% CI 48, 62) compared to 300 mg ACN, 150 mg ACN and 0 mg ACN (means were 46 min, 39, 62; 49 min, 42, 57; 48 min, 36, 60, respectively) but this was not statistically significant (P =0.06).

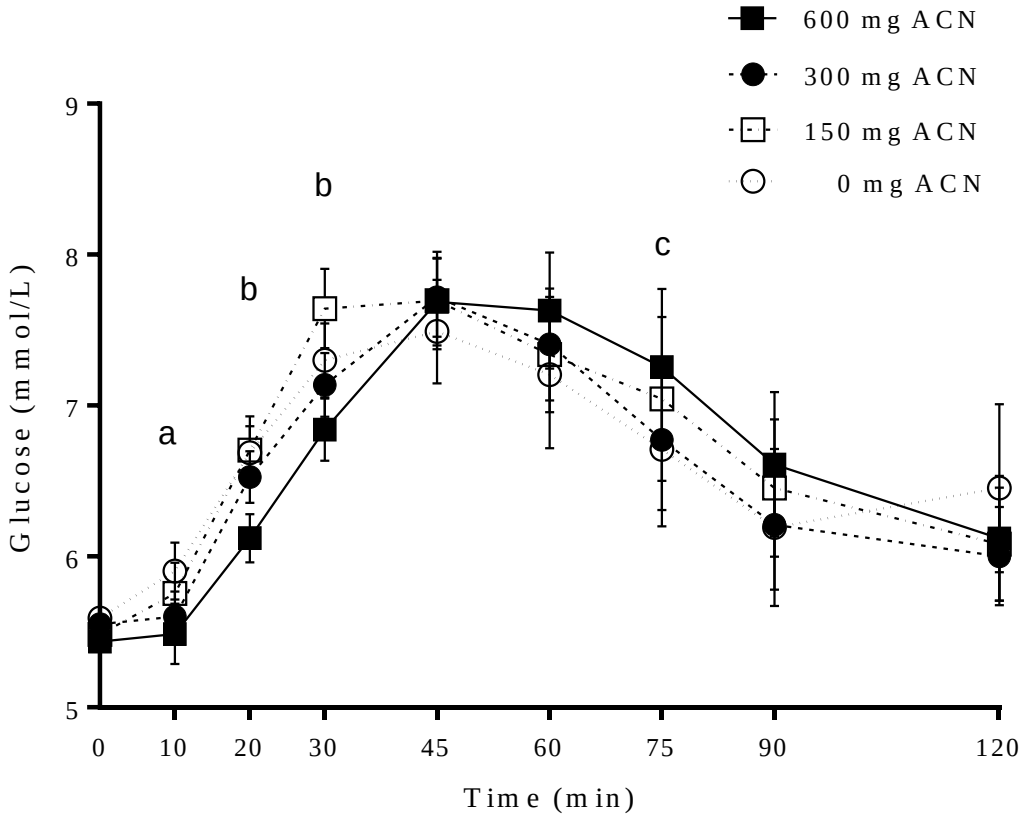


Figure 3.3 Postprandial plasma glucose concentrations

Mean (±SEM) plasma glucose concentrations following ingestion of 4 low sugar fruit drinks 2 minutes before consuming a mixed carbohydrate meal, in randomised order (n=22). All data were natural log transformed before mixed model analysis. There was an overall treatment effect on changes from baseline (P<0.001). Post hoc analysis of time-point differences in change from baseline in glucose compared to 0 mg ACN with Dunnett's adjustment: [a]$P < 0.05$ for the difference between 600 mg ACN and 0 mg ACN and 300 mg ACN and 0 mg ACN; [b]$P < 0.005$ for the difference between 600 mg ACN and 0 mg ACN; [c]$P < 0.01$ for the difference between 600 mg ACN and 0 mg ACN.

There was a statistically significant treatment effect (P <0.001) and treatment x time interaction (P <0.05) for (Ln)insulin 0-120 min, and a treatment x time interaction (P <0.05) for the change from baseline in (Ln)insulin 0-120 min. Post hoc analysis showed significantly lower insulin concentrations following 600 mg ACN compared to 0 mg ACN, at 10, 20 and 30 min, and higher concentrations at 75 and 90 min (**Figure 3.4**). The mean difference in AOB 0-30 min between 600 mg ACN and 0 mg ACN (95% CI) was -8.77 mU/L.h (-13.86, -3.68), P <0.005 **(Table 3.7)**. There were no differences in Cmax, Tmax and AOB 0-120 min for insulin.

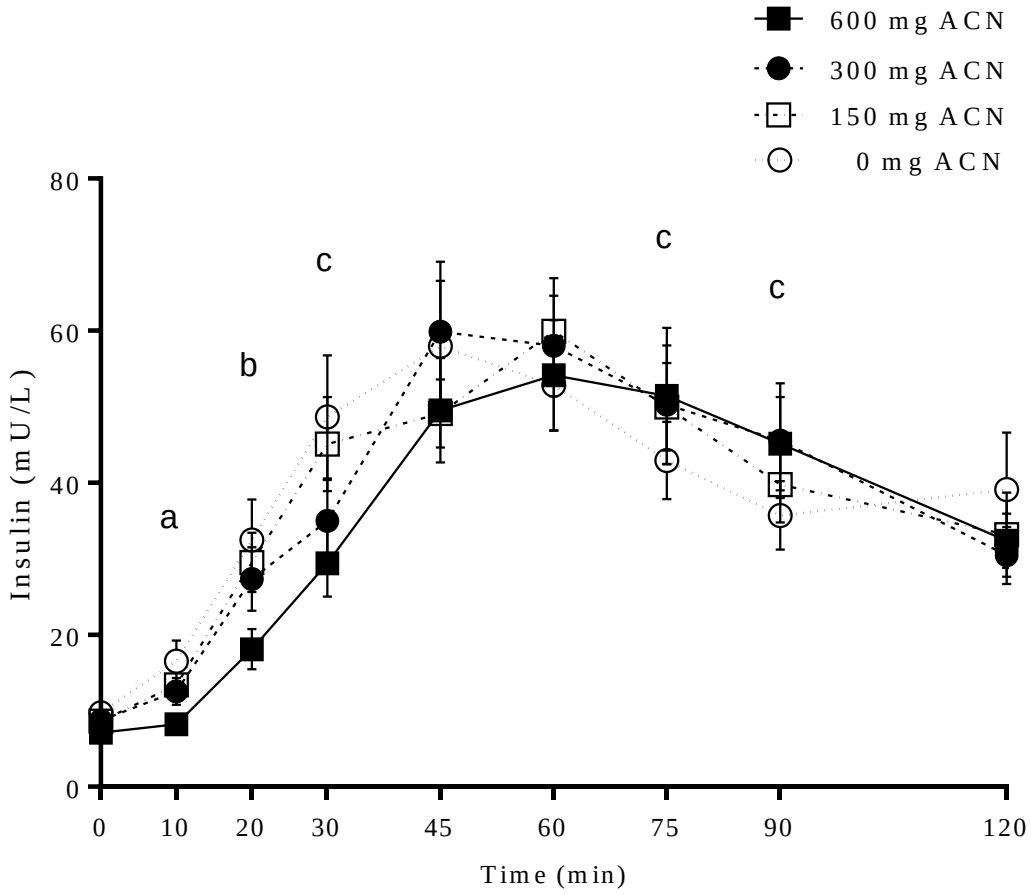


Figure 3.4 Postprandial plasma insulin concentrations
Mean (±SEM) insulin concentrations following ingestion of 4 low sugar fruit drinks 2 minutes before consuming a mixed carbohydrate meal, in randomised order (n=22). All data were natural log transformed before mixed model analysis. There was a treatment effect (P < 0.001) and a treatment x time interaction on raw values and changes from baseline (P < 0.001). Post hoc analysis of time-point differences in change from baseline in insulin with Dunnett's adjustment: [a]P < 0.005 for the difference between 600 mg ACN and 0 mg ACN; [b]P < 0.01 for the difference between 600 mg ACN and 0 mg ACN; [c]P < 0.05 for the difference between 600 mg ACN and 0 mg ACN.

Table 3.7 Effects of blackcurrant extract and placebo test drinks on area over baseline (AOB) 0-30 min for plasma glucose and insulin in GLU-BERRY study population (n=22)

AOB (0-30 min)	**Treatment comparison**	**Mean difference**	**95% CI[1]**	***P* –value[1]**
Glucose (mmol/L·h)	600 mg ACN – 0 mg ACN	-0.34	-0.56, -0.11	0.0049
	600 mg ACN – 150 mg ACN	-0.41	-0.63, -0.18	0.0005
	600 mg ACN – 300 mg ACN	-0.18	-0.41, 0.04	0.1066
Insulin (mU/L·h)	600 mg ACN – 0 mg ACN	-8.77	-13.86, -3.68	0.0011
	600 mg ACN – 150 mg ACN	-7.34	-12.08, -2.59	0.0031
	600 mg ACN – 300 mg ACN	-4.17	-8.86, 0.52	0.0801

[1]Confidence interval and *P*-value adjusted by Dunnett's procedure for multiple comparisons against a control group (0 mg ACN). There were no significant differences between the lower doses (300, 150 mg anthocyanins) relative to placebo. AOB (0-30 min; mmol/L.h) for glucose, 0 mg ACN 0.77 (0.58, 0.96), 150 mg ACN 0.84 (0.66, 1.03), 300 mg ACN 0.62 (0.43, 0.81), 600 mg ACN 0.44 (0.25, 0.63); for insulin, 0 mg ACN 16.56 (11.76, 21.36), 150 mg ACN 15.13 (10.49, 19.78), 300 mg ACN 11.97 (7.40, 16.54), 600 mg ACN 7.80 (3.08, 12.51).

There was statistically significant treatment effect (P<0.0000001) and treatment x time interaction (P<0.005) on (Ln)GIP 0-120 min and change from baseline of (Ln)GIP 0-120 min (treatment effect P<0.00005). Plasma GIP changes from baseline concentration were significantly reduced during the overall postprandial period following 600 mg ACN compared to 0 mg ACN, with a decrease in Cmax (mean difference -64.4 ng/L; -95,5, -33.4; P <0.0005) and AOB 0-120 min (P <0.0001). Post hoc pairwise comparisons with Dunnett's adjustment for multiple comparisons showed reduced plasma GIP concentrations at all time-points following 600 mg ACN up until 90 min (**Figure 3.5**).

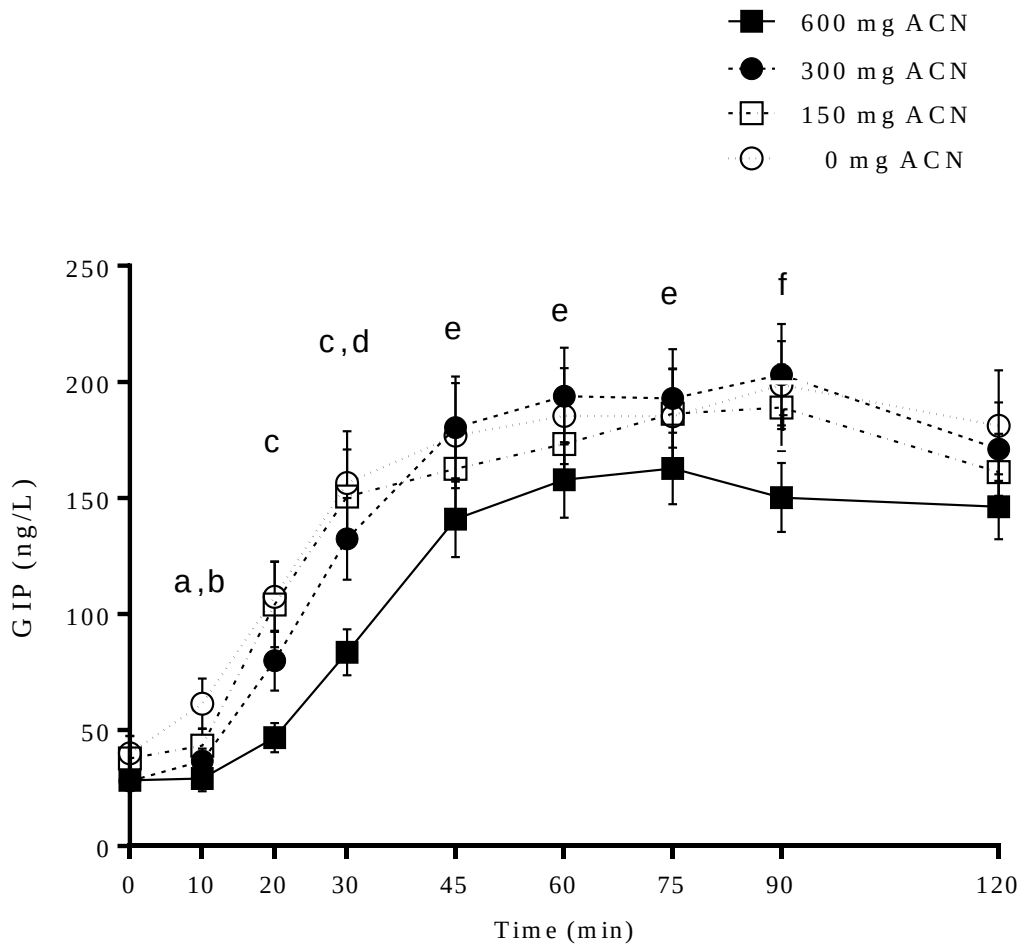


Figure 3.5 Postprandial plasma GIP concentrations

Mean (±SEM) plasma GIP concentrations following ingestion of 4 low sugar fruit drinks 2 minutes before consuming a mixed carbohydrate meal, in randomised order: 600 mg ACN (n=17); 300 mg ACN (n=20); 150 mg ACN (n=20); 0 mg ACN (n=19). All data were natural log transformed before mixed model analysis. There was an overall treatment effect on raw values ($P < 0.0000001$) and changes from baseline ($P < 0.00005$), and a treatment x time interaction on raw values ($P < 0.005$). Post hoc analysis of time-point differences in change from baseline with Dunnett's adjustment: $^{a}P < 0.0001$ for the difference between 600 mg ACN and 0 mg ACN, and $^{b}P < 0.001$ for the difference between 150 mg ACN and 0 mg ACN; $^{c}P < 0.0005$ for the difference between 600 mg ACN and 0 mg ACN; $^{d}P < 0.005$ for the difference between 600 mg ACN and 150 mg ACN and 600 mg ACN and 300 mg ACN; $^{e}P < 0.05$ for the difference between 600 mg ACN and 0 mg ACN; and $^{f}P < 0.0005$ for the difference between 600 mg ACN and 0 mg ACN.

Plasma GLP-1 concentrations were reduced at later time points by 600 mg ACN (**Figure 3.6**), with a statistically significant treatment effect on changes from baseline of (*Ln*)GLP-1 ($P<0.0005$), but no significant treatment x time interaction. Post hoc pairwise comparisons showed reduced plasma GLP-1 concentrations relative to baseline following 600 mg ACN compared to 0 mg ACN and 150 mg ACN anthocyanins at 90 min. There were no differences in Cmax, Tmax and AOB 0-120 min for GLP-1.

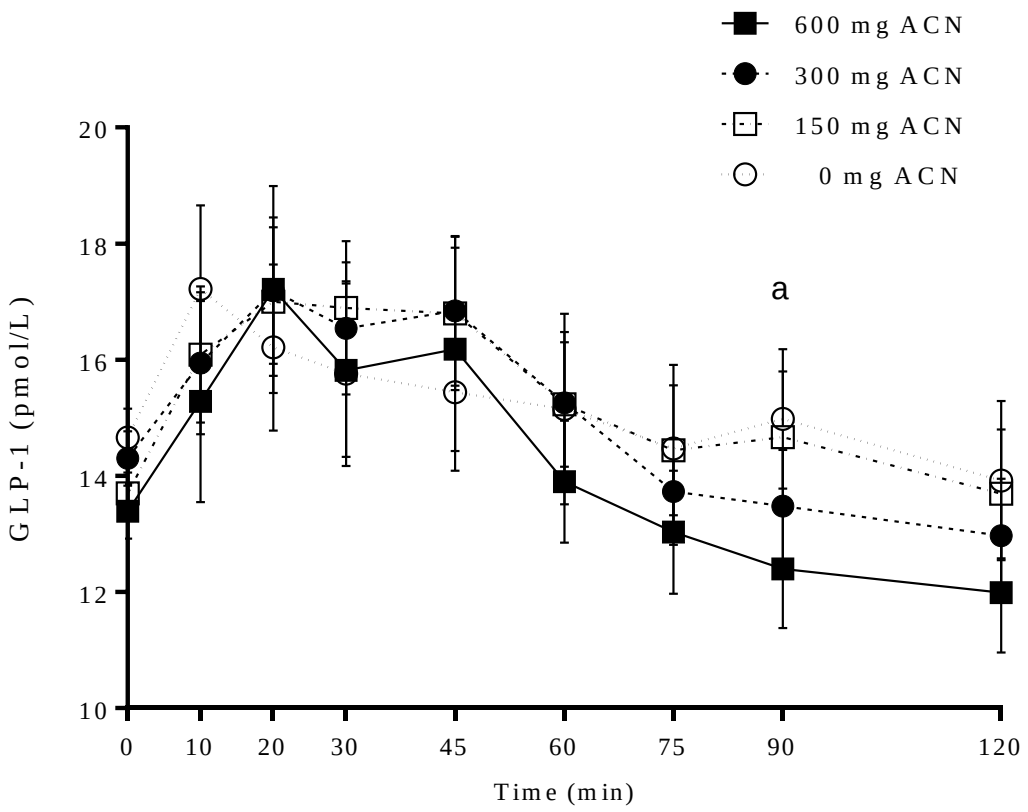


Figure 3.6 Postprandial plasma GLP-1 concentrations
Mean (±SEM) plasma GLP-1 concentrations following ingestion of 4 low sugar fruit drinks 2 minutes before consuming a mixed carbohydrate meal, in randomised order: 600 mg ACN (n=20); 300 mg ACN (n=22); 150 mg ACN (n=21); 0 mg ACN (n=22). All data were natural log transformed before mixed model analysis. There was an overall treatment effect on raw values and changes from baseline ($P < 0.001$). Post hoc analysis of time-point differences in change from baseline with Dunnett's adjustment: $^{a}P < 0.05$ for the difference between 600 mg ACN and 0 mg ACN.

Lipids

Plasma TAG concentrations ((*Ln*)TAG up to 120 min) were significantly different according to treatment but this was due to a significant difference at baseline ($P<0.005$) and the changes from baseline (*Ln*)TAG did not differ (**Figure 3.7**). There was no effect of treatment on plasma NEFA concentrations (**Figure 3.8**).

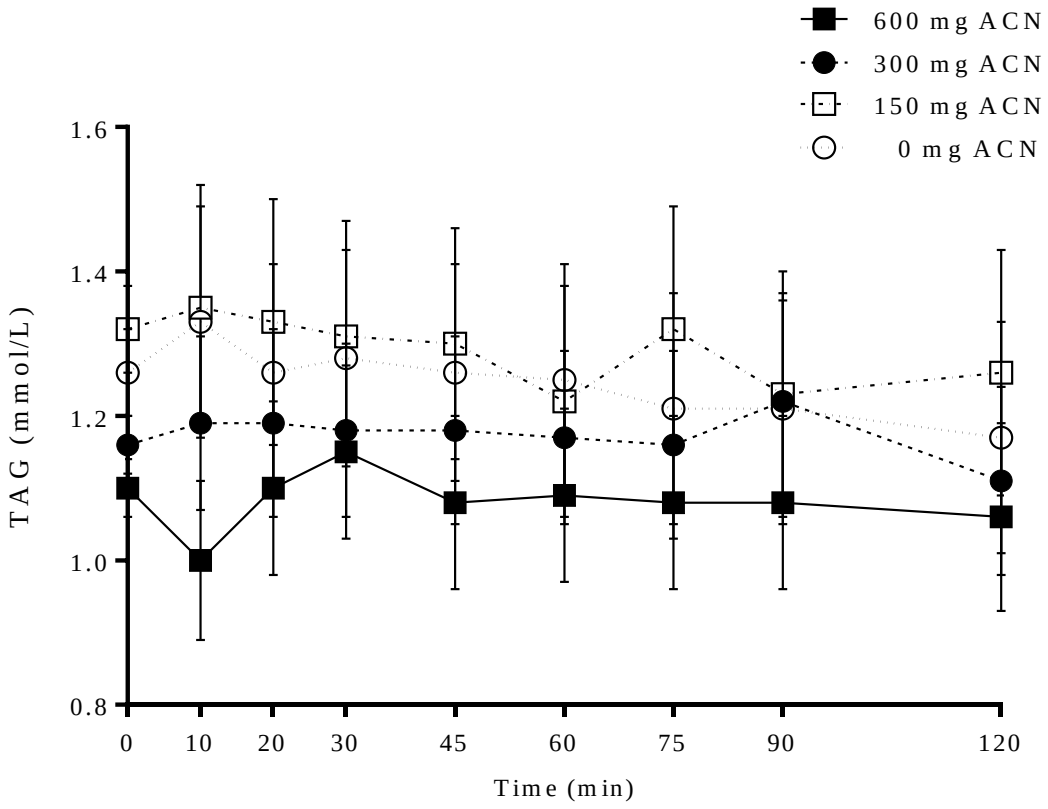


Figure 3.7 Postprandial plasma TAG concentrations
Mean (±SEM) plasma TAG concentrations following ingestion of 4 low sugar fruit drinks 2 minutes before consuming a mixed carbohydrate meal, in randomised order (n=22). All data were natural log transformed before mixed model analysis. There was a treatment effect ($P < 0.0000001$) on raw values but not for changes from baseline and no treatment x time interactions was observed.

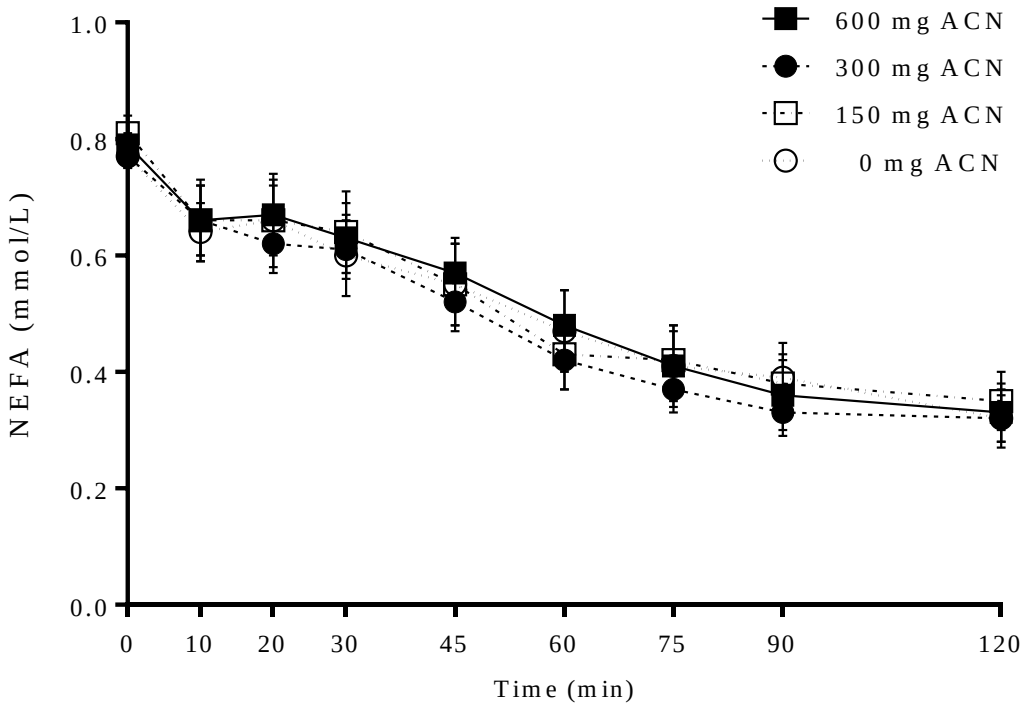


Figure 3.8 Postprandial plasma NEFA concentrations
Mean (±SEM) plasma NEFA concentrations following ingestion of 4 low sugar fruit drinks 2 minutes before consuming a mixed carbohydrate meal, in randomised order (n = 22). All data were natural log transformed before mixed model analysis. There were no statistically significant treatment differences in NEFA concentrations.

Vascular function and isoprostanes

There were no differences between treatments for changes from baseline in DVP-RI, DVP-SI, systolic and diastolic blood pressure and 8-isoprostanes $F_{2\alpha}$ concentrations (**Table 3.8**).

Table 3.8 Effects of blackcurrant extract and placebo test drinks on digital volume pulse (DVP), systolic and diastolic blood pressure (SBP and DBP) and plasma 8-isoprostane $F_{2\alpha}$ in GLU-BERRY study population (n=22)

Variable[1]		**0 mg**	**150 mg**	**300 mg**	**600 mg**
DVP-RI (%)	Baseline	74.4 (69.5, 79.2)	70.4 (64.8, 76.1)	72.9 (67.1, 78.6)	72.8 (68.0, 77.5)
	Δ 120 min	-0.57 (-5.35, 4.21)	3.80 (-0.93, 8.54)	0.15 (-4.26, 4.56)	1.38 (-3.28, 6.04)
DVP-SI (m/s)	Baseline	8.57 (7.75, 9.40)	8.50 (7.63, 9.37)	8.44 (7.64, 9.25)	8.71 (7.62, 9.80)
	Δ 120 min	-0.75 (-1.44, -0.05)	-0.23 (-1.05, 0.59)	-0.57 (-1.21, 0.07)	-0.55 (-1.54, 0.45)
SBP (mmHg)	Baseline	120.8 (116.1, 125.2)	120.9 (115.6, 126.0)	120.7 (116.0, 125.5)	118.3 (113.7, 122.9)
	Δ 120 min	-4.5 (-8.8, -0.1)	-1.0 (-6.8, 4.9)	-5.2 (-8.9, -1.5)	-1.7 (-6.7, 3.3)
DBP (mmHg)	Baseline	74.6 (71.2, 78.1)	75.3 (71.7, 78.9)	74.5 (71.3, 77.8)	73.6 (70.8, 76.4)
	Δ 120 min	-0.6 (-4.3, 3.2)	1.7 (-2.0, 5.4)	-1.1 (-3.6, 1.3)	0.5 (-2.5, 3.5)
Plasma isoprostane (pmol/L)[2]	Baseline	86.4 (68.8, 108.4)[3]	123.7 (96.6, 158.3)[3]	100.5 (80.8, 125.1)	98.4 (76.6, 126.2)
8-isoprostanes $F_{2\alpha}$	Δ 120 min	1.13 (1.01, 1.27)	0.94 (0.81, 1.09)	1.02 (0.91, 1.14)	0.97 (0.85, 1.11)

[1]Values are mean baseline values and changes from baseline measured 120 min after the test drink and mixed carbohydrate meal, (95% CI), n=22.
[2]Values are geometric mean baseline values and log ratios of changes from baseline measured 120 min after the test drink and mixed carbohydrate meal, (95% CI), n=22. For example, the log ratio of change from baseline in 8-isoprostanes $F_{2\alpha}$ following placebo was 1.13, equating to a 13% increase from baseline.
[3] n=20 due to sample loss

3.5.2 GLU-APP study

A total of 27 healthy men and postmenopausal women aged 20 to 60 years attended screening sessions; all of them met inclusion criteria and therefore were found eligible to participate in the study. Twenty two participants were randomised to treatment and 22 completed the GLU-APP study. Details of study stages are shown on the consort diagram (**Figure 3.9**) and characteristics of the 22 participants who completed the study are shown in **Table 3.9**.

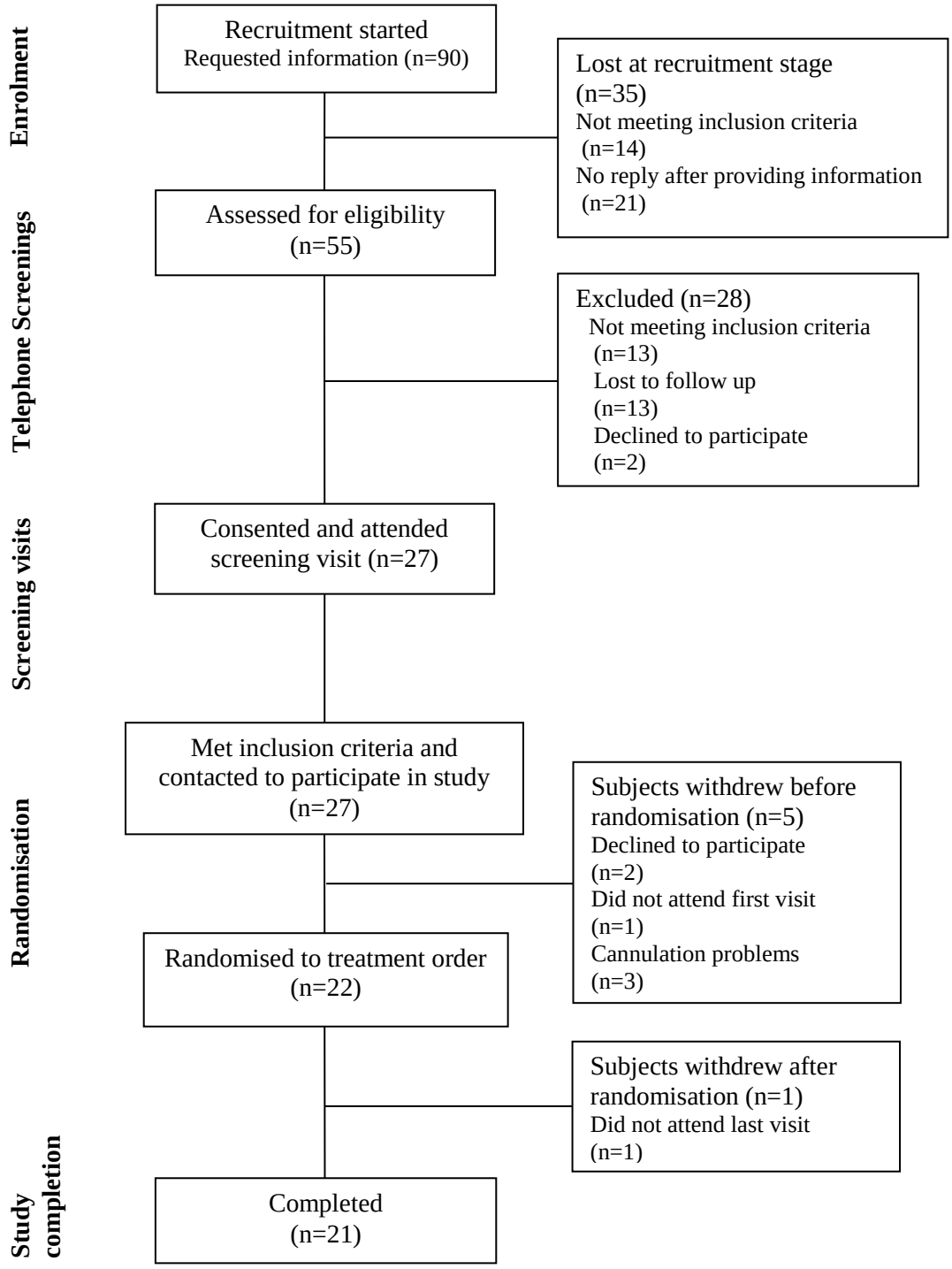


Figure 3.9 Consort diagram of GLU-APP study

Table 3.9 Characteristics of GLU-APP study population (n=22)

Variable [1]	
Age (y)	41.5 (13.3)
Sex (male to female ratio)	14:8
Body Mass Index (kg/m^2)	24.9 (3.7)
Systolic blood pressure (mmHg)	118.6 (18.0)
Diastolic blood pressure (mmHg)	79.7 (10.4)
Waist circumference (cm)	
Males	87.8 (6.0)
Females	93.0 (14.0)
Body fat (%)	
Males	17.8 (4.7)
Females	35.8 (6.8)
Fasting plasma glucose (mmol/L)	5.0 (0.4)
Fasting plasma triacylglycerol (mmol/L)	1.2 (0.9)
Fasting plasma total cholesterol (mmol/L)	5.3 (1.2)
Fasting plasma LDL cholesterol (mmol/L)	3.2 (0.9)
Fasting plasma HDL cholesterol (mmol/L)	
Males	1.3 (0.2)
Females	1.9 (0.5)

[1]Values are means (SD)

There was no effect of treatment on plasma glucose and plasma insulin concentrations. Plasma concentrations of glucose and insulin 0-120 min are shown in **Figures 3.10** and **3.11**. There were no differences for change from baselines, Cmax, Tmax or AOB **(Table 3.10)** on plasma glucose and plasma insulin concentrations. The analysis of time-points suggested no dose response effect over time.

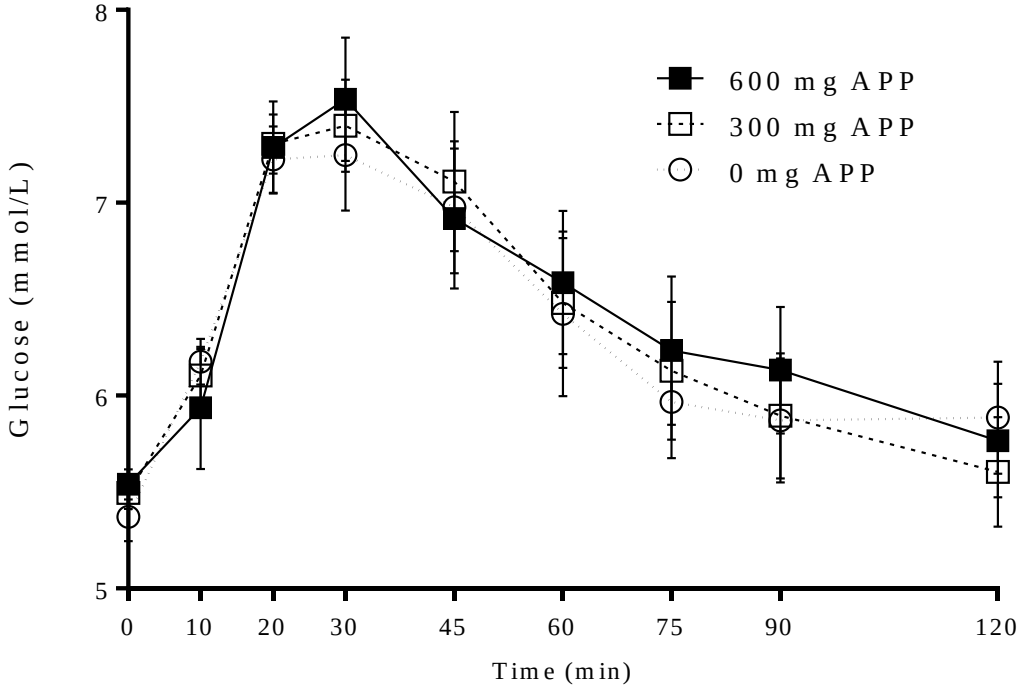


Figure 3.10 Postprandial plasma glucose concentrations
Mean (±SEM) plasma glucose concentrations following ingestion of 3 low sugar fruit drinks 2 minutes before consuming a high carbohydrate meal, in randomised order (n=22). There were no statistically significant differences in glucose concentrations between treatments.

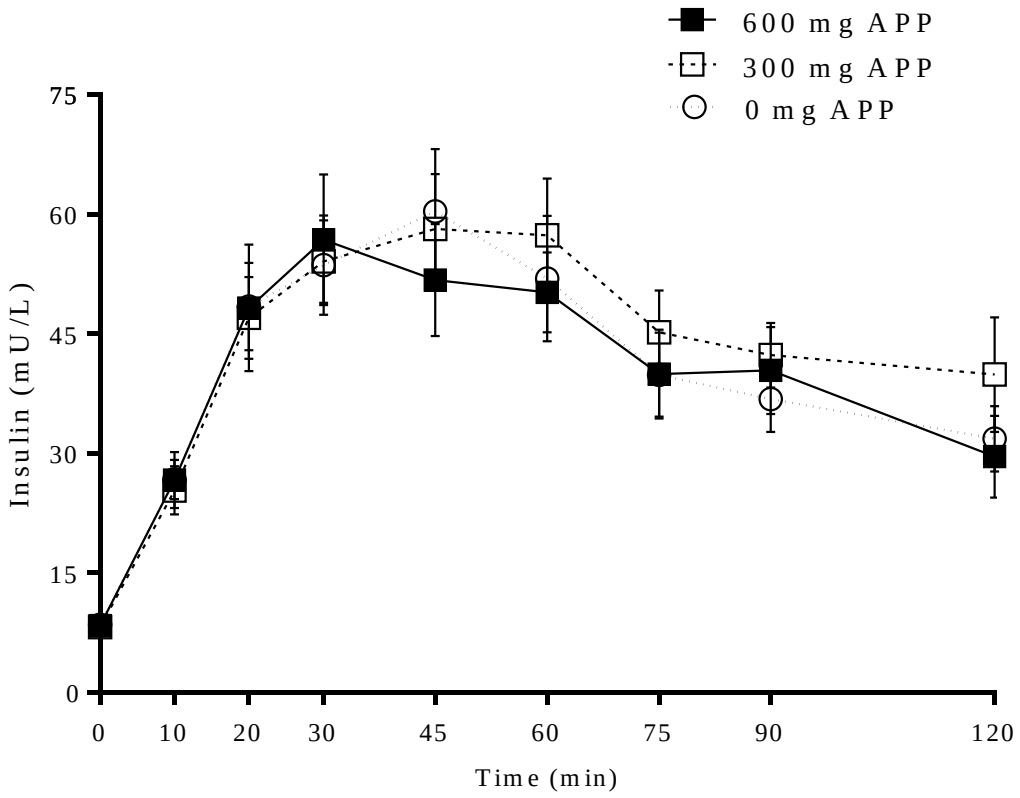


Figure 3.11 Postprandial plasma insulin concentrations
Mean (±SEM) plasma insulin concentrations following ingestion of 3 low sugar fruit drinks 2 minutes before consuming a high carbohydrate meal, in randomised order: 600 mg APP, 600 mg of apple polyphenols; 300 mg APP, 300 mg of apple polyphenols and 0 mg APP, 0 mg apple polyphenols (control), (n=22). There were no statistically significant differences in insulin concentrations between treatments.

There was no effect of treatment on plasma GIP and GLP-1 concentrations in 0-120 min postprandial period. There were no differences for changes from baseline concentrations for any of the plasma concentrations. However post hoc pairwise comparisons showed elevated plasma GLP-1 concentration relative to baseline following 600 mg APP compared to 0 mg APP at 20 min (**Figure 3.12**). There were no differences in Cmax, Tmax and AOB 0-120 min for GIP and GLP-1 (**Table 3.10**).

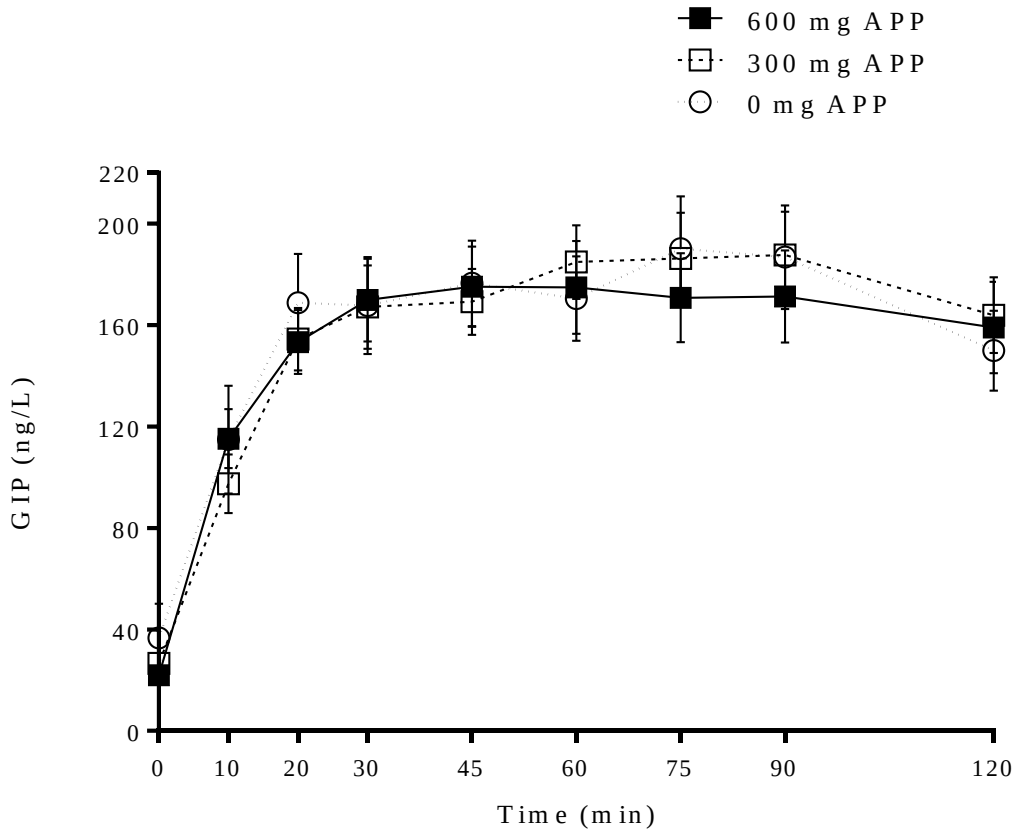


Figure 3.12 Postprandial plasma GIP concentrations
Mean (±SEM) plasma GIP concentrations following ingestion of 3 low sugar fruit drinks 2 minutes before consuming a high carbohydrate meal, in randomised order (n=22). There were no statistically significant differences in GIP concentrations between treatments.

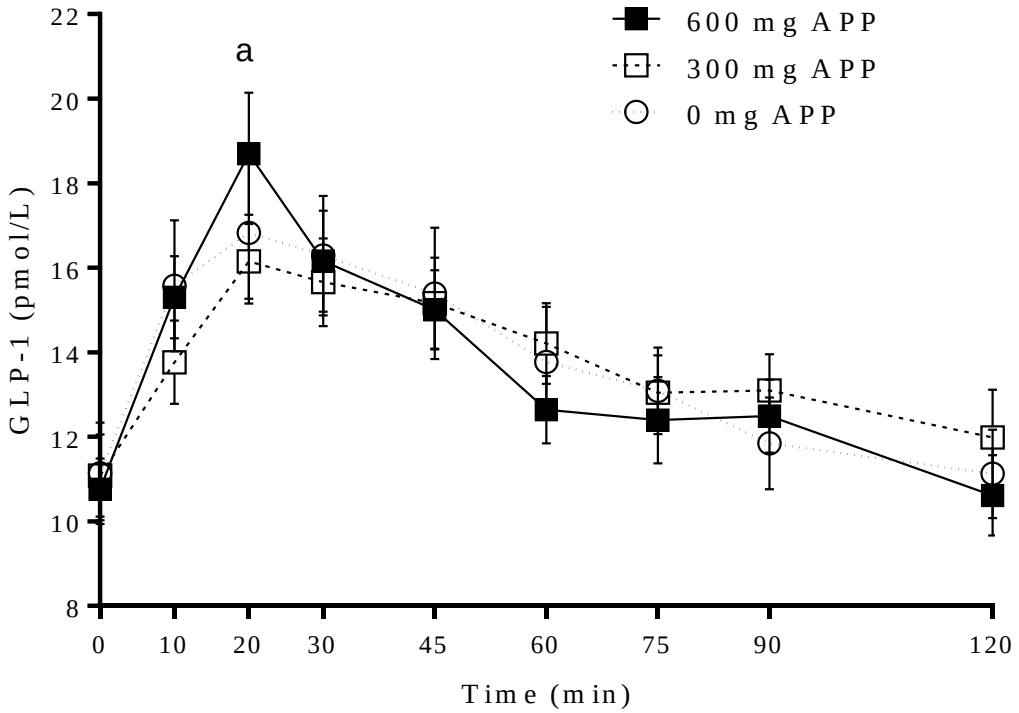


Figure 3.13 Postprandial plasma GLP-1 concentrations
Mean (±SEM) plasma GLP-1 concentrations following ingestion of 3 low sugar fruit drinks 2 minutes before consuming a high carbohydrate meal, in randomised order (n=22). There were no statistically significant differences in GIP concentrations between treatments. Post hoc analysis with Dunnet's adjustment: [a]*P*= 0.048 for the difference between 600 mg APP and 0 mg APP.

Lipids

There was no effect of treatment on plasma TAG and NEFA concentrations in 0-120 min postprandial period. There were no differences for changes from baseline concentrations for any of the plasma concentrations. There were no differences in Cmax, Tmax and AOB 0-120 min for TAG and NEFA (**Table 3.10**).

Table 3.10 Effects of apple extract and placebo test drinks on area over baseline (AOB) 0-120 min for plasma glucose, insulin, GIP, GLP-1, TAG and NEFA concentrations in GLU-APP study population (n=22)

AOB[1] (0-120 min)	**Treatment**	**Mean**	**95% CI**[1]
Glucose (mmol/L·min)	0 mg APP	0.98	(0.42, 1.54)
	300 mg APP	0.88	(0.40, 1.36)
	600 mg APP	1.19	(0.46, 1.92)
Insulin (mU/L·min)	0 mg APP	34.7	(27.8, 41.6)
	300 mg APP	36.8	(29.5, 44.0)
	600 mg APP	32.9	(25.3, 40.6)
GIP (ng/L·min)	0 mg APP	127.5	(101.9, 153.2)
	300 mg APP	135.2	(108.2, 162.2)
	600 mg APP	139.8	(107.4, 172.2)
GLP-1 (pmol/L·min)	0 mg APP	2.22	(1.12, 3.32)
	300 mg APP	2.71	(1.43, 3.99)
	600 mg APP	2.93	(1.73, 4.13)
TAG (mmol/L·min)	0 mg APP	-0.08	(-0.19, 0.03)
	300 mg APP	-0.03	(-0.07, 0.01)
	600 mg APP	-0.04	(-0.08, 0.00)
NEFA (mmol/L·min)	0 mg APP	-0.29	(-0.37, -0.21)
	300 mg APP	-0.32	(-0.39, -0.25)
	600 mg APP	-0.31	(-0.39, -0.23)

600 mg APP, 600 mg of apple polyphenols; 300 mg APP, 300 mg of apple polyphenols; 0 mg APP, 0 mg apple polyphenols (control).

[1] From ANCOVA model with treatment and period as fixed factors, subject as random effect and subject baseline and period baseline as covariates (n=22).

Vascular function and isoprostanes

There were no differences between treatments for changes from baseline in DVP-RI, DVP-SI and systolic and diastolic blood pressure (**Table 3.11**). Isoprostanes were not analysed due to a shortage of the 8-Isoprostane affinity columns from supplier.

Table 3.11 Effects of apple extract and placebo test drinks on digital volume pulse (DVP) and systolic and diastolic blood pressure (SBP and DBP) in GLU-APP study population (n=22)

Variable[1]		**0 mg**	**300 mg**	**600 mg**
DVP-RI (%)	Baseline	59.3 (54.4, 64.2)	57.1 (50.1, 64.1)	58.6 (51.2, 66.0)
	Δ 120 min	1.14 (-2.92, 5.20)	-1.21 (-6.40, 3.99)	0.01 (-4.23, 4.25)
DVP-SI (m/s)	Baseline	6.7 (6.1, 7.4)	6.8 (6.1, 7.6)	7.0 (6.3, 7.8)
	Δ 120 min	0.02 (-0.21, 0.25)	-0.11 (-0.43, 0.21)	0.08 (-0.19, 0.35)
SBP (mmHg)	Baseline	118.9 (113.9, 123.9)	120.5 (114.5, 126.6)	122.5 (117.2, 127.8)
	Δ 120 min	-0.52 (-3.54, 2.49)	-1.06 (-3.90, 1.78)	1.51 (-0.18, 3.21)
DBP (mmHg)	Baseline	72.6 (69.5, 75.7)	74.3 (70.4, 78.3)	73.5 (69.5, 77.5)
	Δ 120 min	-0.10 (-1.54. 1.34)	0.41 (-2.08, 2.90)	-0.29 (-2.04, 1.46)

There were no differences between treatments for changes from baseline
[1]Values are mean baseline values and changes from baseline measured 120 min after the test drink and mixed carbohydrate meal, (95% CI), n=22.

3.5.3 GLU-FRU study

A total of 42 healthy men and postmenopausal women aged 20 to 60 years attended screening sessions, 34 meet all inclusion criteria and therefore were found eligible to participate in the study. Participants were randomised to treatment and 25 subjects completed the GLU-FRU study. Details of study stages are shown on the consort diagram (**Figure 3.14**) and characteristics of the 25 participants who completed the study are shown in **Table 3.12**.

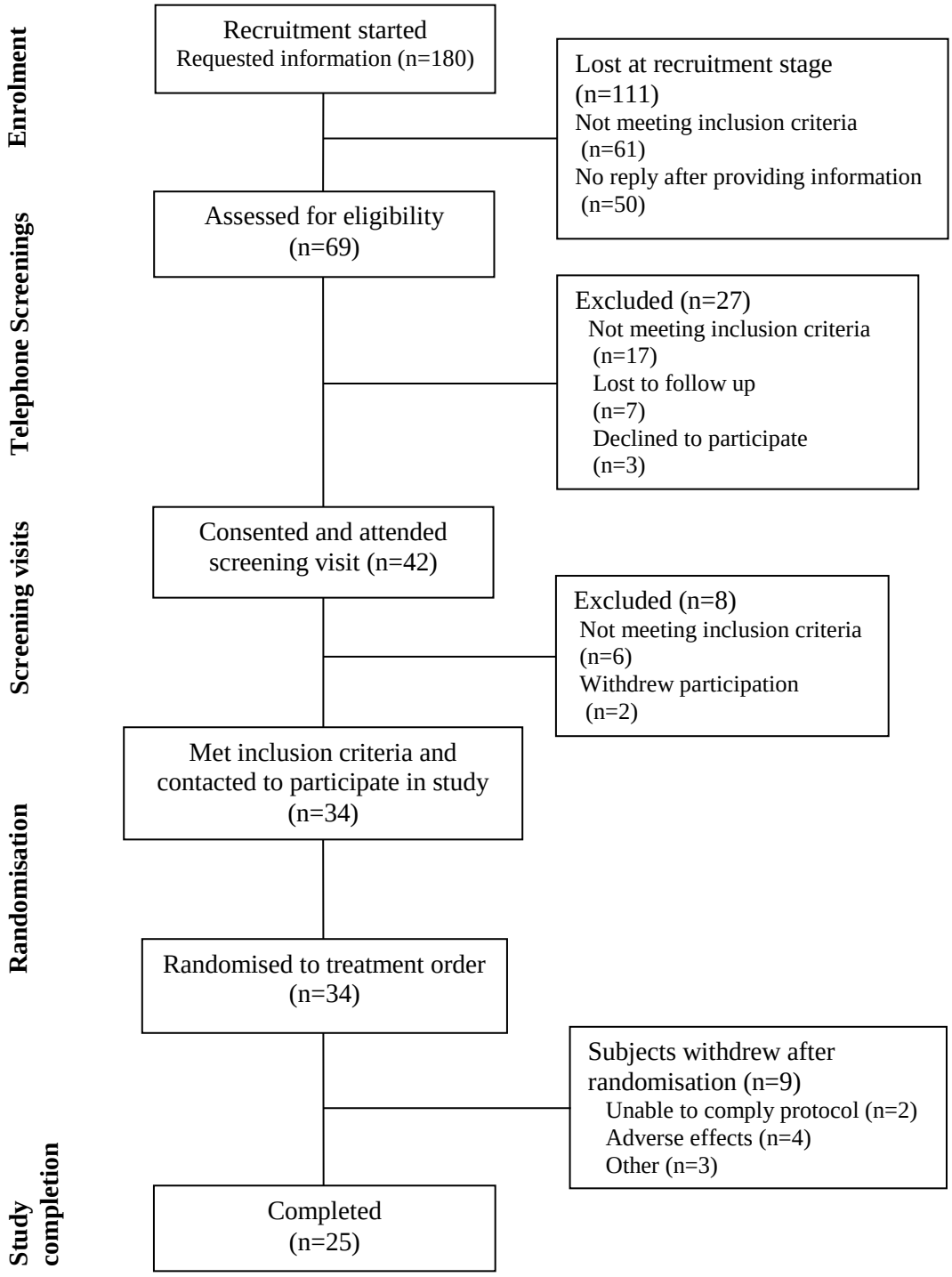


Figure 3.14 Consort diagram of GLU-FRU study

Table 3.12 Characteristics of GLU-FRU study population (n=25)

Variable [1]	
Age (y)	32.3 (14.4)
Sex (male to female ratio)	20:5
Body Mass Index (kg/m^2)	23.5 (2.8)
Systolic blood pressure (mmHg)	112.2 (10.8)
Diastolic blood pressure (mmHg)	69.2 (7.9)
Waist circumference (cm)	
Males	81.9 (7.4)
Females	85.8 (11.4)
Body fat (%)	
Males	16.4 (5.8)
Females	34.9 (5.5)
Fasting plasma glucose (mmol/L)	5.1 (0.3)
Fasting plasma triacylglycerol (mmol/L)	1.1 (0.9)
Fasting plasma total cholesterol (mmol/L)	4.3 (1.0)
Fasting plasma LDL cholesterol (mmol/L)	2.6 (0.9)
Fasting plasma HDL cholesterol (mmol/L)	
Males	1.3 (0.3)
Females	1.6 (0.4)

[1]Values are means (SD)

There was an overall treatment effect for the change from baseline of glucose concentrations 0-120 min (P=0.003), with statistically significant treatment x time interaction for postprandial glucose response (P=0.000). As **Figure 3.15** illustrates, AE+BE treatment inhibited glucose concentrations during the 120 min of the postprandial period, change from baseline 0-120 min mean difference AE+BE vs CON (95% CI) -0.52 mmol/L (-0.86,-0.18), P=0.002. iAUC 0-120 min was significantly different between treatments, mean differences AE+BE vs CON (95% CI) -51.6 mmol/L.min (-93.8, -9.4), P=0.014. iAUC 0-30 min was statistically significant different between treatments, AE+BE and AE treatments significantly lower glucose concentrations compared to CON, see **Table 3.14** for details. Post hoc analysis with Tukey's adjustment, showed significantly lower glucose concentrations following AE and AE+BE compared to CON at 10, 20 and 30 min post-drink, and lower glucose concentration for AE at 60 min. Cmax was significantly different between treatments, AE and AE+BE had a lowering effect on maximal plasma glucose concentration when compared to CON. Cmax mean difference AE vs CON (95% CI) -0.7 (-1.3, -0.1), P=0.023; Cmax mean difference AE+BE vs CON (95% CI) -0.9 (-1.6, -0.3), P=0.002. Tmax was significantly different between treatments, was greater following AE+BE (median at 60 min, IQR 45, 75, P=0.000) compared to AE and CON (medians were 45 min, 37, 60 and 30 min, 20, 30, respectively), see **Table 3.13** for details.

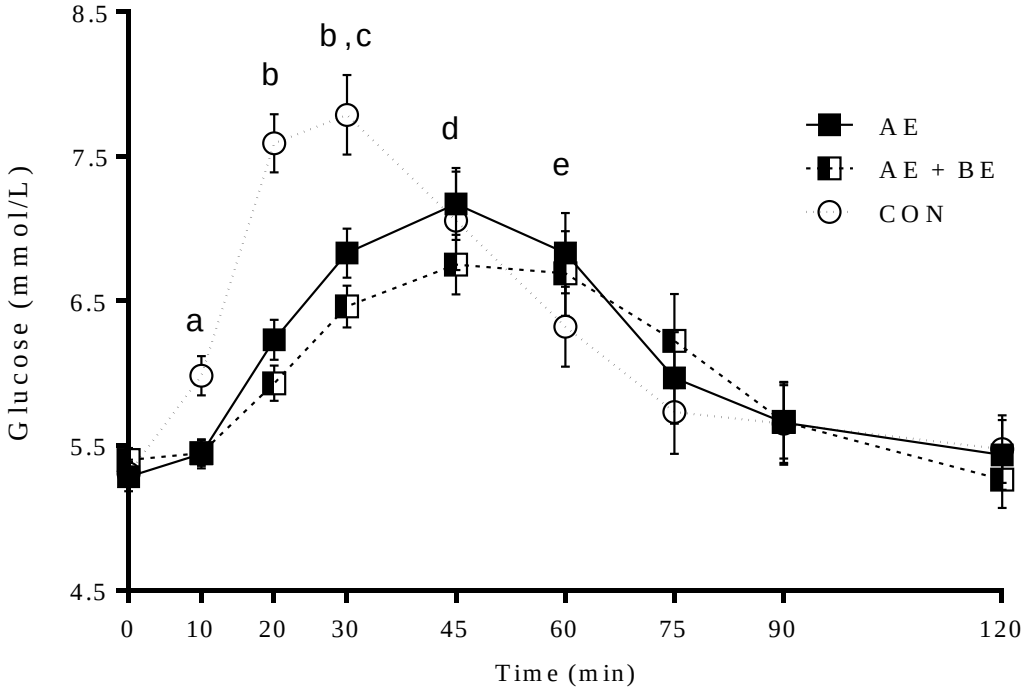


Figure 3.15 Postprandial glucose concentrations

Mean (±SEM) plasma glucose concentrations following ingestion of 3 low sugar fruit drinks 2 minutes before consuming a mixed carbohydrate meal, in randomised order (n = 25). AE; 1200 mg of apple polyphenols, AE+BE; 600 mg of apple polyphenols + 600 mg blackcurrant anthocyanins, CON; 0 mg of blackcurrant anthocyanins and apple polyphenols. There was an overall treatment effect on changes from baseline (P=0.003). Post hoc analysis of time-point differences in change from baseline in glucose with Tukey's adjustment: [a]$P < 0.05$ for the difference between CON and AE and AE+BE; [b]$P<0.0001$ for the difference between CON and AE and AE+BE; [c]$P < 0.001$ for the difference between AE and AE+BE and CON; [d]$P < 0.05$ for the difference between AE and AE+BE; [e]$P < 0.05$ for the difference between CON and AE.

There was an overall treatment effect for the change from baseline of (*Ln*)insulin concentrations 0-120 min ($P<0.0005$), with a statistically significant treatment x time interaction for postprandial insulin response ($P<0.0001$) (**Figure 3.16)**. Both treatments, AE+BE and AE inhibited insulin concentrations during the 120 min of the postprandial period, change from baseline 0-120 min mean difference AE+BE vs CON (95% CI) -0.40 mIU/L (-0.64,-0.16), $P<0.0005$, and mean difference AE vs CON (95% CI) -0.27 mIU/L (-0.51,-0.03), $P<0.05$. iAUC 0-120 min was significantly different between treatments, mean difference AE+BE vs CON (95% CI) -1109.7 mIU/L·min (-1642.2, -577.2), $P=0.000$ and mean difference AE vs CON (95% CI) -872.7 mIU/L·min (-1734.4,-11.1), $P=0.047$. iAUC 0-30 min was statistically significantly different between treatments, both AE+BE and AE treatments significantly lowered insulin concentrations compared to CON, see **Table 3.14** for details. Post hoc analysis for multiple comparisons with Tukey's adjustment, showed significantly lower (*Ln*)insulin concentrations following AE and AE+BE compared to CON at 10, 20, 30 and 45 min post-drink and AE vs CON at 120 min. Cmax was significantly different between treatments, AE+BE had a lowering effect on maximal plasma (*Ln*)insulin concentration when compared to CON. Cmax mean difference AE+BE vs CON (95% CI) -16.8 (-33.5, -0.11), $P=0.048$. Tmax was significantly different between treatments, being greater following AE+BE (median at 75 min, IQR 67, 75, $P=0.000$) compared to AE and CON (medians were 60 min, 45, 75 and 45 min, 25, 60, respectively), see **Table 3.13** for details.

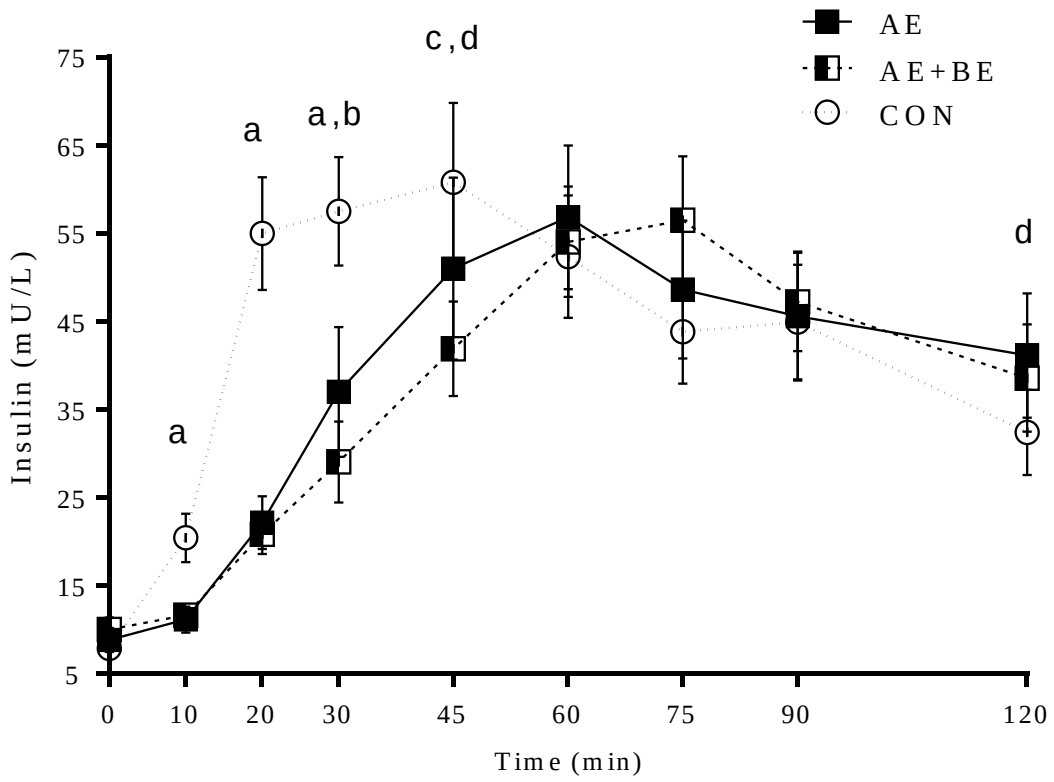


Figure 3.16 Postprandial insulin concentrations
Mean (±SEM) plasma insulin concentrations following ingestion of 3 low sugar fruit drinks 2 minutes before consuming a mixed carbohydrate meal, in randomised order (n = 25). AE; 1200 mg of apple polyphenols, AE+BE; 600 mg of apple polyphenols + 600 mg blackcurrant anthocyanins, CON; 0 mg of blackcurrant anthocyanins and apple polyphenols. There was an overall treatment effect on changes from baseline ($P < 0.001$). Post hoc analysis of time-point differences in change from baseline in insulin compared with Tukey's adjustment: [a]$P < 0.0001$ for the difference between CON and AE and AE+BE; [b]$P<0.005$ for the difference between AE and AE+BE; [c]$P<0.0001$ for the difference between CON and AE+BE; [d]$P<0.05$ for the difference between CON and AE.

There was an overall treatment effect for the change from baseline of (*Ln*)C-peptide concentrations 0-120 min ($P<0.0001$), with statistically significant treatment x time interaction for postprandial C-peptide response ($P<0.0001$) (**Figure 3.17)**. Treatments, AE+BE and AE suppressed C-peptide concentrations during the 120 min of the postprandial period, change from baseline 0-120 min mean difference AE+BE vs CON (95% CI) -0.29 pmol/L (-0.40,-0.18), $P<0.0001$, and mean difference AE vs CON (95% CI) -0.16 pmol/L (-0.27,-0.05), $P=0.004$. iAUC 0-120 min was significantly different between treatments, mean difference AE+BE vs CON (95% CI) -33779.1 pmol/L·min (-48831.4, -18726.9), $P=0.000$. iAUC 0-30 min was statistically significant different between treatments, both AE+BE and AE treatments significantly lower C-peptide concentrations compared to CON, see **Table 3.14** for details. Post hoc analysis with Tukey's adjustment for multiple comparisons, showed significantly lower C-peptide concentrations following AE and AE+BE compared to CON at 10, 20, 30 and 45 min post-drink and AE+BE vs CON at 60 min. Cmax was not significantly different between treatments. Tmax was greater following AE+BE and AE (medians were 75 min, IQR 60, 90, and 60 min, 60, 105, respectively, $P=0.000$) compared to CON (median at 60 min, 45, 75), see **Table 3.13** for details.

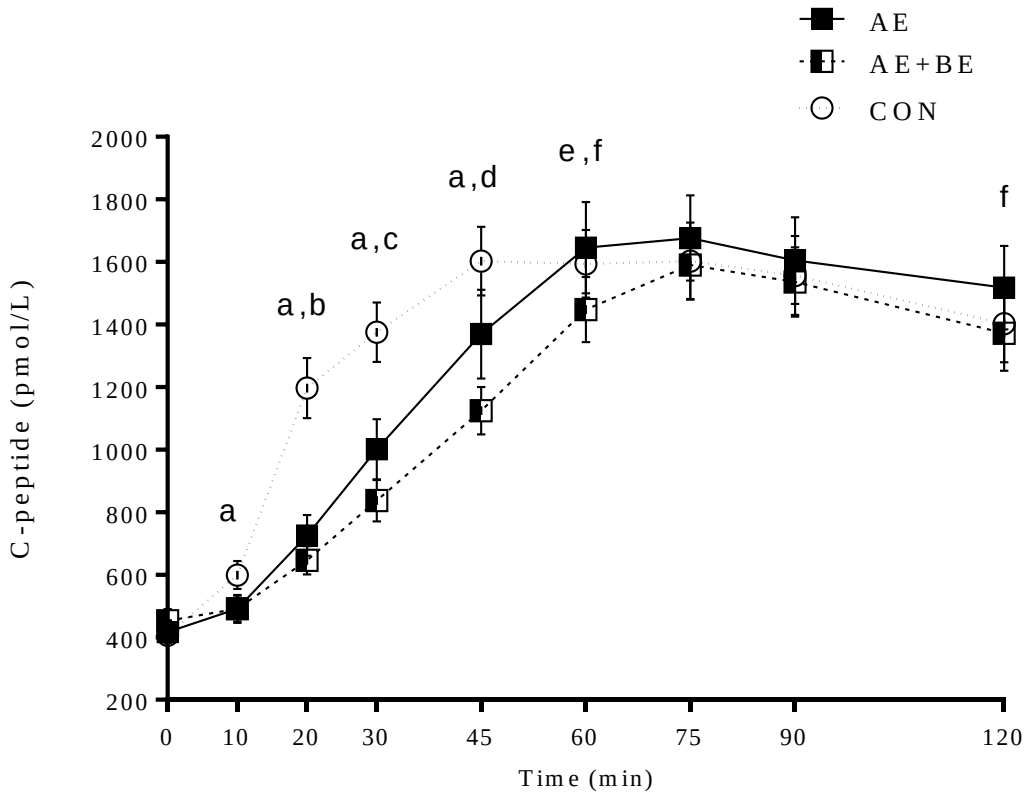


Figure 3.17 Postprandial C-peptide concentrations
Mean (±SEM) plasma C-peptide concentrations following ingestion of 3 low sugar fruit drinks 2 minutes before consuming a mixed carbohydrate meal, in randomised order (n = 25). AE; 1200 mg of apple polyphenols, AE+BE; 600 mg of apple polyphenols + 600 mg blackcurrant anthocyanins, CON; 0 mg of blackcurrant anthocyanins and apple polyphenols. There was an overall treatment effect on changes from baseline (P<0.0001). Post hoc analysis of time-point differences in change from baseline in C-peptide with Tukey's adjustment: [a]P < 0.0001 for the difference between CON and AE and AE+BE; [b]P<0.05 for the difference between AE and AE+BE; [c]P<0.0001 for the difference between AE and AE+BE; [d]P<0.0005 for the difference between AE and AE+BE; [e]P<0.005 for the difference between CON and AE+BE; [f]P<0.05 for the difference between AE and AE+BE .

Plasma GIP changes from baseline concentration were significantly reduced during the whole postprandial period by treatments AE and AE+BE compared to CON, with a statistically significant treatment effect (P=0.0001) and treatment x time interaction (P<0.0001) on change from baseline of (*Ln*)GIP 0-120 min (**Figure 3.18**). Treatments, AE+BE and AE inhibited plasma GIP concentrations along the 120 min of postprandial period, change from baseline 0-120 min mean difference AE+BE vs CON (95% CI) -0.55 ng/L (-0.83,-0.26), P=0.000, and mean difference AE vs CON (95% CI) -0.36 ng/L (-0.65,-0.07), P=0.010. iAUC 0-120 min was significantly different between treatments, mean difference AE+BE vs CON (95% CI) -6097.2 ng/L·min (-7742.5, -4451.9), P=0.000; mean difference AE vs CON (95% CI) -4038.1 ng/L·min (-5467.5 -2608.8), P=0.000. iAUC 0-30 min was statistically significant different between treatments, both AE+BE and AE treatments significantly lower GIP concentrations compared to CON, see **Table 3.14** for details. Post hoc analysis with Tukey's adjustment for multiple comparisons, showed significantly lower GIP concentrations following AE and AE+BE compared to CON at all timepoints up to 75 min. There was a significant decrease in Cmax following AE+BE and AE vs CON (mean differences -45.5 ng/L; (95% CI) (-73.6, -17.5), P=0.003 and -32.7 ng/L; (-54.1, -11.4), P=0.005). Tmax was greater following BE+AE (median at 90 min, IQR 82, 120, P=0.000) compared to AE and CON (medians were 90 min, 75, 120 and 60 min, 45, 75, respectively), see **Table 3.13** for details.

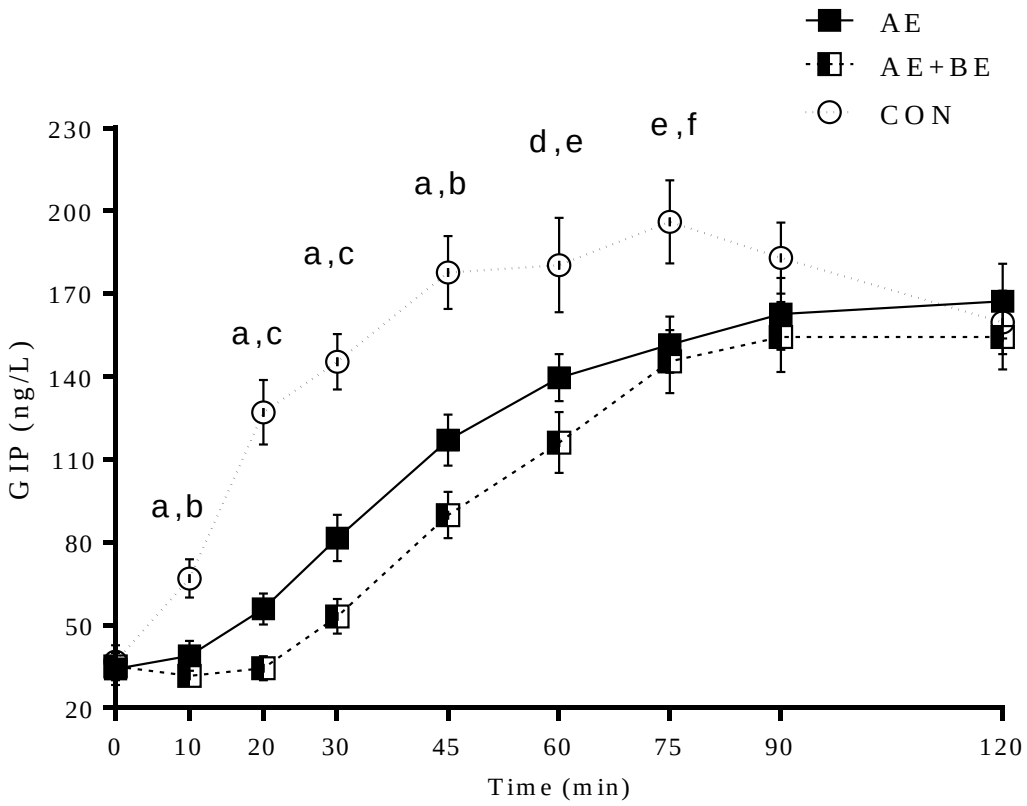


Figure 3.18 Postprandial GIP concentrations
Mean (±SEM) plasma GIP concentrations following ingestion of 3 low sugar fruit drinks immediately before consuming a mixed carbohydrate meal, in randomised order (n = 25). AE; 1200 mg of apple polyphenols, AE+BE; 600 mg of apple polyphenols + 600 mg blackcurrant anthocyanins, CON; 0 mg of blackcurrant anthocyanins and apple polyphenols. There was an overall treatment effect on changes from baseline (P=0.0001). Post hoc analysis of time-point differences in change from baseline with Tukey's adjustment: [a]P<0.0001 for the difference between CON and AE and AE+BE; [b]P<0.05 for the difference between AE and AE+BE, [c]P<0.0001 for the difference between AE and AE+BE; [d]P<0.0001 for the difference between CON and AE+BE; [e]P<0.05 for the difference between CON and AE; [f]P<0.05 for the difference between CON and AE+BE.

Incremental area under the curve (iAUC) 0-30 min for plasma glucose, insulin, C-peptide and GIP were significantly different between treatments. Both treatments AE and AE+BE decreased the early response of variables involved in the glycaemic response when compared with control. Due to technical problems with the GLP-1 assay conducted at Kings College Hospital results are not available.

Table 3.13 Incremental area under the curve (iAUC), maximum concentration (Cmax) and time of maximum concentration (Tmax) for plasma glucose, insulin, C-peptide and GIP concentrations following consumption of apple, apple and blackcurrant and placebo test drinks and a starch and sucrose meal (n=25).

	Glucose	**Insulin**	**C-peptide**	**GIP**
iAUC[1] (0-30 min)	mmol/L·min	mIU/L·min	pmol/L·min	ng/L·min
CON	42.16 (16.3)	845.6 (468.6)	14660.4 (6337.1)	1745.7 (640.5)
AE	19.03 (9.5)	297.3 (304.8)	6749.8 (4569.5)	502.3 (347.0)
AE+BE	11.23 (8.0)	220.4 (159.9)	3713.4 (3137.3)	66.9 (324.3)
P (main treatment effect)	<0.0001	<0.0001	<0.0001	<0.0001
iAUC[1] (0-120 min)	mmol/L·min	mIU/L·min	pmol/L·min	ng/L·min
CON	119.40 (117.8)	4423.2 (2544.1)	116419.2 (40664.0)	14336.4 (4748.3)
AE	103.90 (101.9)	3758.5 (3119.2)	104543.1 (50677.6)	10331.6 (3705.3)
AE+BE	76.26 (101.9)	3476.8 (2019.3)	87006.0 (33614.1)	8406.5 (4245.6)
P (main treatment effect)	0.029	0.028	<0.0001	<0.0001
Cmax[1]	mmol/L	mIU/L	pmol/L	ng/L
CON	8.2 (1.3)	80.0 (47.2)	1895.2 (684.2)	210.9 (79.9)
AE	7.6 (1.2)	69.6 (54.0)	1871.2 (739.9)	180.4 (67.7)
AE+BE	7.3 (1.2)	69.0 (35.1)	1728.1 (531.9)	170.4 (64.5)
P (main treatment effect)	<0.0001	0.240	0.135	0.001
Tmax[2] (min)				
CON	30 (20, 30)	45 (25, 60)	60 (45, 75)	60 (45, 75)
AE	45 (37, 60)	60 (45, 75)	75 (60, 105)	90 (75, 120)
AE+BE	60 (45, 75)	75 (60, 75)	75 (60, 90)	90 (82, 120)
P (main treatment effect)	<0.0001	<0.0001	<0.0001	<0.0001

[1] All values are Mean (SD)

[2] All valueas are Median (IQR)

Two-way repeated measures analysis of variance (ANOVA) were used to test for statistically significant treatment effects, apart from Tmax where a non-parametric related samples Friedman's test was used.

Table 3.14 Effects of apple, apple and blackcurrant and placebo test drinks in incremental area under the curve (iAUC) 0-30 min for plasma glucose, insulin, C-peptide and GIP in GLU-FRU study population (n=25)

iAUC (0-30 min)	Treatment comparison	Mean difference	95% CI[1]	*P* -value[1]
Glucose (mmol/L·min)	AE – CON	-26.3	-34.9, -17.7	0.000
	AE+BE – CON	-31.5	-40.8, -22.2	0.000
	AE+BE – AE	-5.2	-9.8, -0.6	0.023
Insulin (mU/L·min)	AE – CON	-613.1	-771.6, -454.6	0.000
	AE+BE – CON	-675.3	-915.1, -435.5	0.000
	AE+BE – AE	-62.2	-203.0, 78.6	0.782
C-peptide (pmol/L·min)	AE – CON	-9221.9	-11031.5, -7142.3	0.000
	AE+BE – CON	-11881.4	-15099.3, -8663.6	0.000
	AE+BE – AE	-2659.5	-4817.5, -501.6	0.013
GIP (ng/L·min)	AE – CON	-1272.7	-1568.3, -977.2	0.000
	AE+BE – CON	-1735.3	-1998.1, -1472.6	0.000
	AE+BE – AE	-462.6	-617.2, -307.9	0.000

AE; 1200 mg of apple polyphenols, AE+BE; 600 mg of apple polyphenols + 600 mg blackcurrant anthocyanins, CON; 0 mg of blackcurrant anthocyanins and apple polyphenols.
[1] From two-way analysis of variance, confidence interval and *P*-value adjusted by Bonferroni's procedure for multiple comparisons against a reference group (CON), n=25.

Plasma TAG concentrations are showed in **Figure 3.19**; there were no differences between treatments for raw data and change from baseline 0-120 min following the consumption of the test meals.

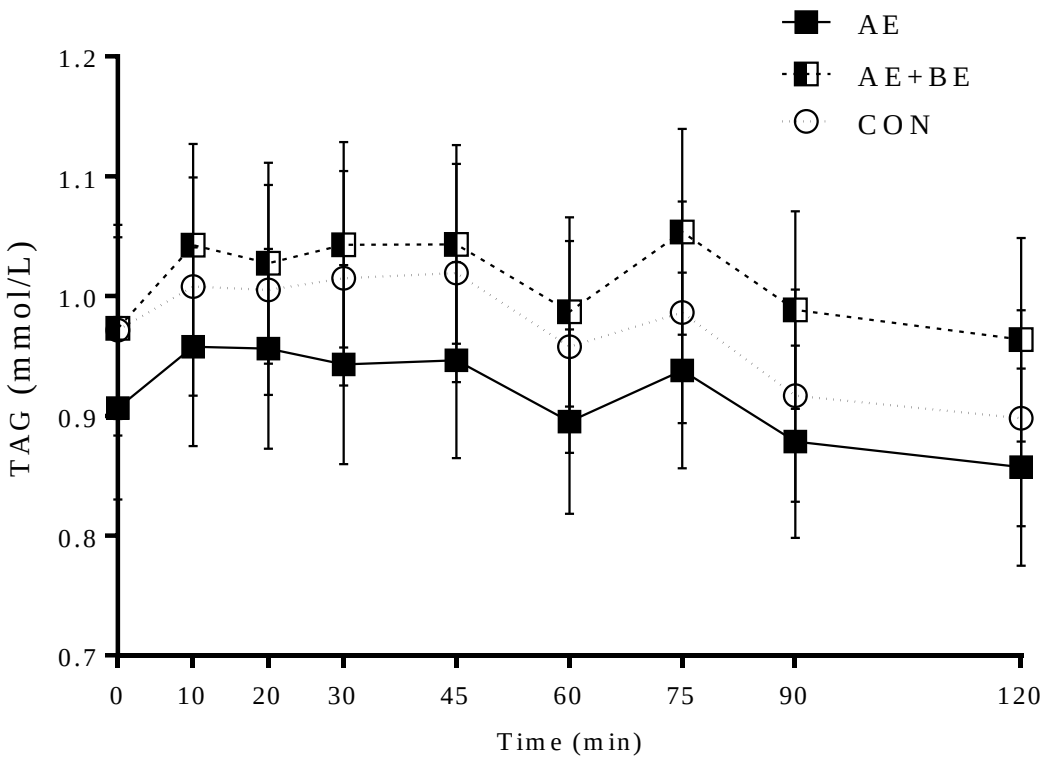


Figure 3.19 Postprandial triacylglycerol concentrations
Mean (±SEM) plasma TAG concentrations following ingestion of 3 low sugar fruit drinks immediately before consuming a mixed carbohydrate meal, in randomised order (n=25). AE; 1200 mg of apple polyphenols, AE+BE; 600 mg of apple polyphenols + 600 mg blackcurrant anthocyanins, CON; 0 mg of blackcurrant anthocyanins and apple polyphenols. There was no statistically significant difference between treatments. All data were natural log transformed analysis.

Decreases in plasma NEFA concentrations relative to baseline were significantly reduced during the whole postprandial period by treatment AE+BE compared to CON, with a statistically significant treatment effect ($P<0.005$) and treatment x time interaction ($P<0.0001$) on change from baseline of NEFA 0-120 min (**Figure 3.20**). Treatment AE+BE inhibited the decreases in plasma NEFA concentrations along the 120 min of postprandial period, change from baseline 0-120 min mean difference AE+BE vs CON (95% CI) 0.12 mmol/L (0.03, 0.20), $P=0.004$. iAUC 0-120 min was significantly different between AE+BE and CON, mean difference (95% CI) 17.2 mmol/L.min (7.2, 27.3), $P=0.001$. Post hoc analysis with Tukey's adjustment for multiple comparisons, showed significantly higher NEFA concentrations following AE and AE+BE compared to CON from 20 min up to 120 min. There was a significant increase in Cmax following AE+BE vs CON (mean differences -0.14 mmol/L; (95% CI) (-0.23, -0.05), $P=0.003$. There were no significant differences in Tmax.

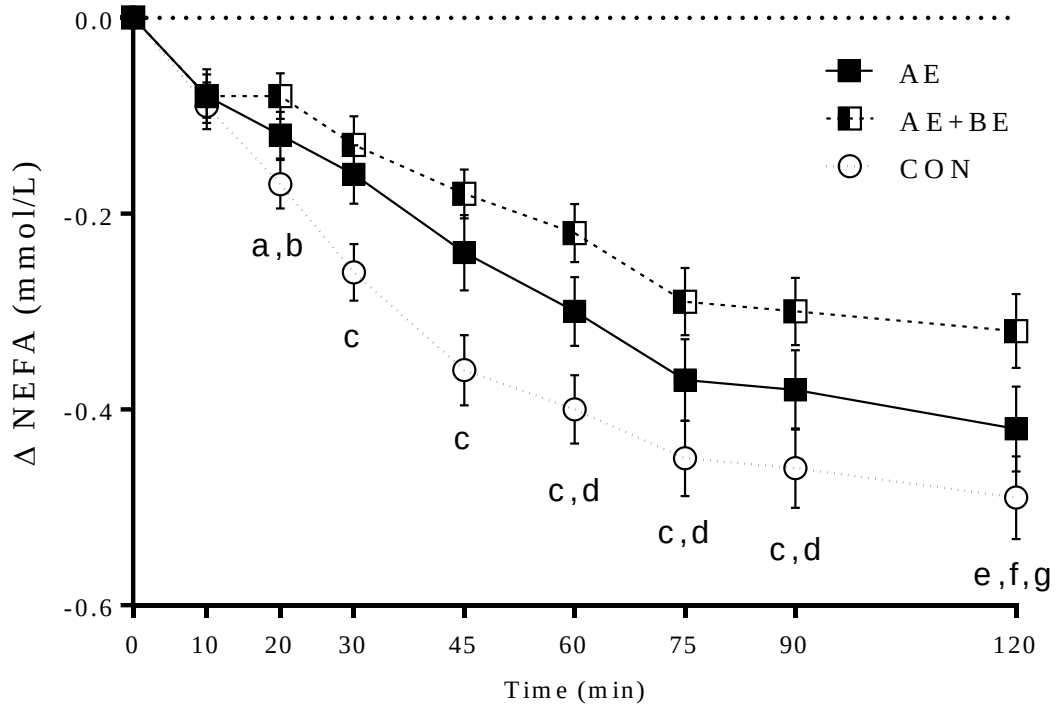


Figure 3.20 Postprandial non-esterified fatty acids concentrations
Baseline values (mmol/L): AE 0.47±0.05 AE+BE 0.40±0.04, CON 0.54±0.04. Mean (±SEM) plasma NEFA concentrations following ingestion of 3 low sugar fruit drinks immediately before consuming a mixed carbohydrate meal, in randomised order (n = 25). AE; 1200 mg of apple polyphenols, AE+BE; 600 mg of apple polyphenols + 600 mg blackcurrant anthocyanins, CON; 0 mg of blackcurrant anthocyanins and apple polyphenols. There was an overall treatment effect on changes from baseline (P=0.000). Post hoc analysis of time-point differences in change from baseline in NEFA with Tukey's adjustment: [a]P<0.0005 for the difference between CON and AE+BE; [b]P<0.05 for the difference between CON and AE, [c]P<0.0001 for the difference between CON and AE and AE+BE, [d]P<0.05 for the difference between AE and AE+BE; [e]P<0.0001 for the difference between CON and AE+BE, [f]P<0.0005 for the difference between CON and AE; [g]P<0.005 for the difference between AE and AE+BE.

There were no differences between treatments on SBP and DBP for raw data or changes from baseline 0-120 min (**Figure 3.21)**.

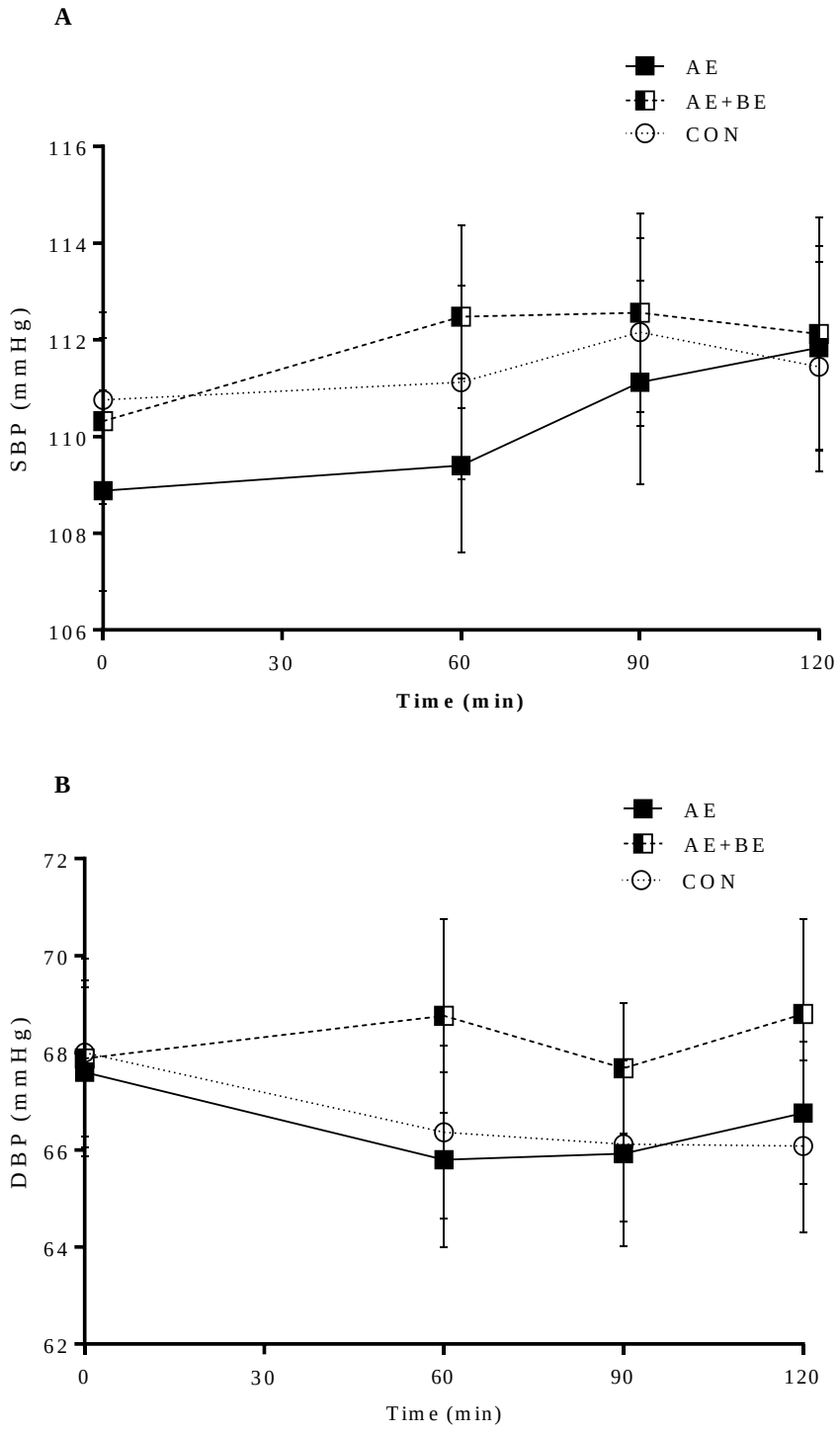


Figure 3.21 Postprandial systolic and diastolic blood pressures values
Mean (±SEM) SBP and DBP values following ingestion of 3 low sugar fruit drinks immediately before consuming a mixed carbohydrate meal, in randomised order (n = 25). AE; 1200 mg of apple polyphenols, AE+BE; 600 mg of apple polyphenols + 600 mg blackcurrant anthocyanins, CON; 0 mg of blackcurrant anthocyanins and apple polyphenols. There was no statistically significant difference between treatments.

There were no differences between treatments for DVP-SI for raw data and change from baseline (**Figure 3.22**).

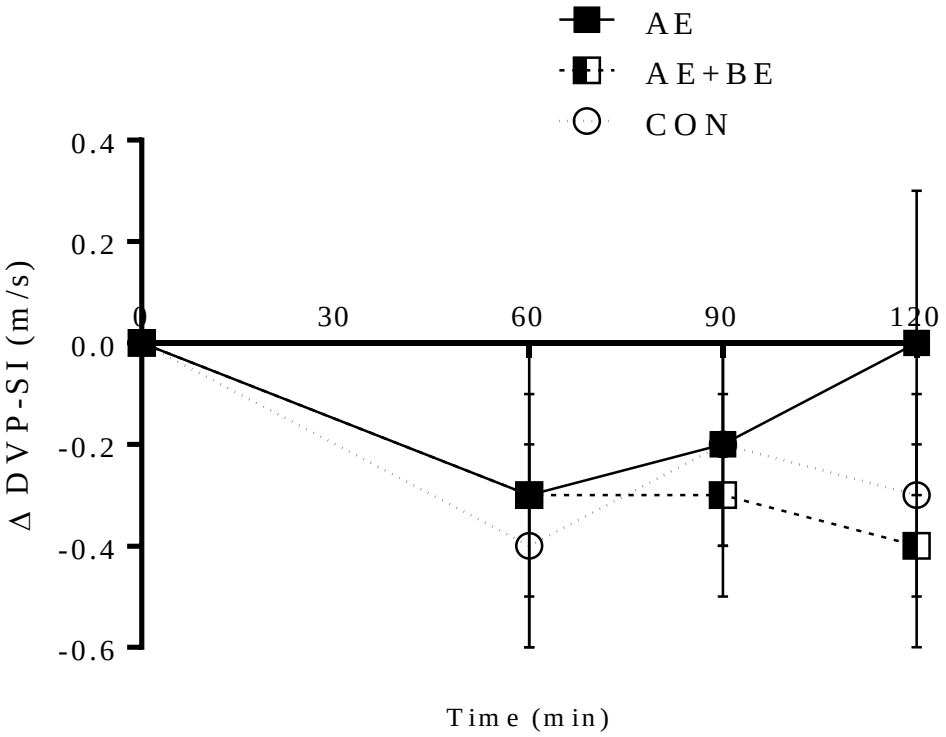


Figure 3.22 Postprandial changes from baseline on stiffness index values
Baseline values (m/sec): AE 6.2, AE+BE 6.5, CON 6.4. Mean (±SEM) DVP-SI values following ingestion of 3 low sugar fruit drinks immediately before consuming a mixed carbohydrate meal, in randomised order (n=25). AE; 1200 mg of apple polyphenols, AE+BE; 600 mg of apple polyphenols + 600 mg blackcurrant anthocyanins, CON; 0 mg of blackcurrant anthocyanins and apple polyphenols. There was no statistically significant difference between treatments.

There were no differences in raw data for reflection index values, however changes from baseline values were significantly reduced during the whole postprandial period by treatment AE compared to CON, with a statistically significant treatment effect (P=0.01) and significant effect on treatment x time interaction 0-120 min (P=0.05) (**Figure 3.23**). Post hoc analysis with Tukey's adjustment for multiple comparisons, showed treatments AE and AE+BE reduced reflection index at 90 and 120 min of postprandial period. Change from baseline 0-120 min mean difference AE vs CON (95% CI) -7.04 m/sec (-12.3, -1.8), P=0.007.

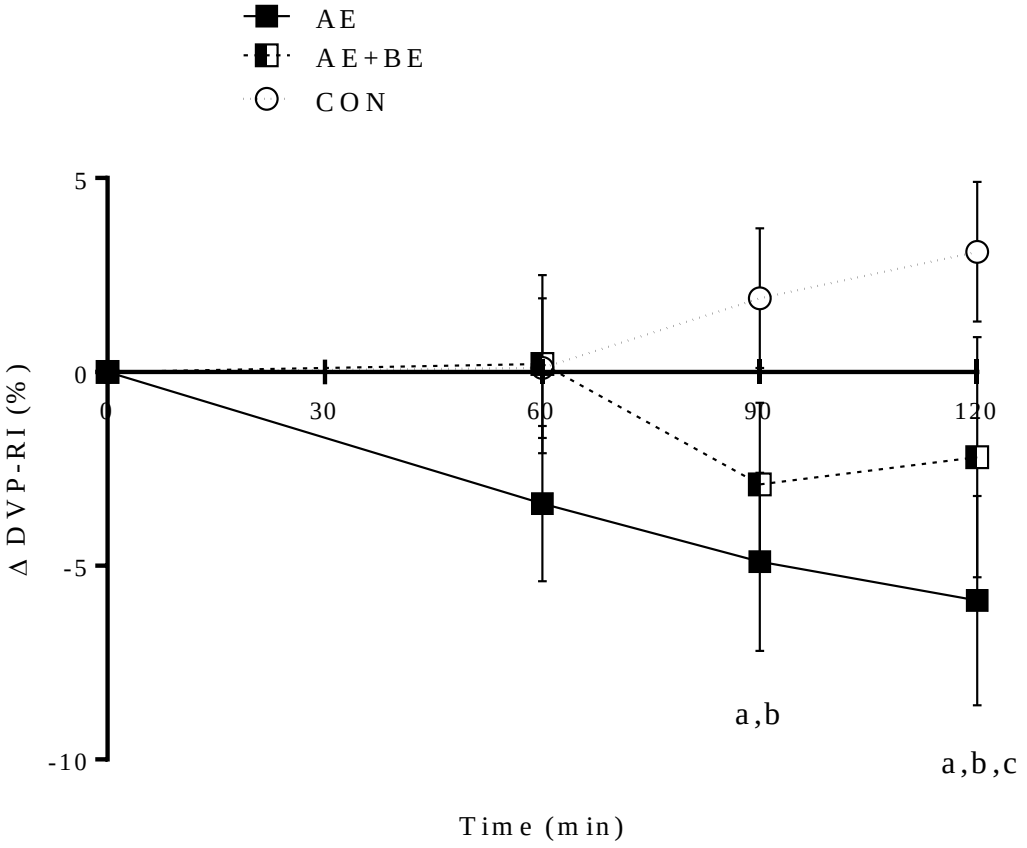


Figure 3.23 Postprandial changes from baseline on reflection index values
Baseline values (%): AE 67, BE+AE 65, CON 63. Mean (±SEM) DVP-RI values following ingestion of 3 low sugar fruit drinks immediately before consuming a mixed carbohydrate meal, in randomised order (n=25). AE; 1200 mg of apple polyphenols, AE+BE; 600 mg of apple polyphenols + 600 mg blackcurrant anthocyanins, CON; 0 mg of blackcurrant anthocyanins and apple polyphenols. There was an overall treatment effect on changes from baseline (P=0.05) following ingestion of treatment AE. Post hoc analysis of time-point differences in change from baseline in DVP-RI with Tukey's adjustment: [a]P<0.0001 for the difference between AE and CON; [b]P<0.05 for the difference between CON and AE+BE; [c]P<0.05 for the difference between AE and AE+BE.

3.6 Discussion

3.6.1 GLU-BERRY study

An inhibitory effect on postprandial glycaemia and insulinaemia was observed following consumption of the highest dose of blackcurrant extract contained in a no added sugar fruit drink. Medium and lowest dose treatments did not show any effect. Early postprandial (0-30 min) plasma glucose and insulin response was decreased by the 600 mg of blackcurrant anthocyanins (600 mg ACN) treatment although a rebound effect was observed at 75 and 90 min. These results coincide with previous findings on studies consuming whole berry meals or berry nectars (juices) rich in anthocyanins, including strawberries, bilberries, cranberries and blackcurrants and showing plasma glucose and insulin concentrations inhibition in the first 30 min following consumption (Törrönen *et al.*, 2012a, Törrönen *et al.*, 2013, Törrönen *et al.*, 2012c, Törrönen *et al.*, 2010, Törrönen *et al.*, 2012b). The present data also agrees with previous work that shows that berries consumed along with a starch-containing test meal have a more marked effect in suppressing insulin concentrations than they do on glucose concentrations (Törrönen *et al.*, 2013).

The rebound effect on blood glucose and insulin concentrations observed at 75 and 90 min might be explained by effects on digestive processing of starch and sucrose contained in our test meal. Diverse polyphenol-rich extracts have been shown to inhibit digestive enzymes involved in the metabolism of complex carbohydrates. Polyphenolic compounds of blackcurrant extract such as proanthocyanidins may reduce pancreatic α-amylase activity in the duodenum (Grussu *et al.*, 2011), anthocyanins and other blackcurrant polyphenols may reduce intestinal brush border α-glucosidases activities (maltase and sucrase) (Akkarachiyasit *et al.*, 2010). Polyphenol-induced delayed digestive processing is likely to have resulted in partially digested carbohydrates possibly flowing further down the small intestine. This may have increased the proportion of glucose that was absorbed later (75-90 min) relative to control accounting for the crossover in glucose and insulin profiles, and this also is in agreement with glycaemic/insulinaemic profiles observed previously (Törrönen *et al.*, 2012a, Törrönen *et al.*, 2012c, Törrönen *et al.*, 2010). Since the 0, 150 and 300 mg ACN intervention drinks also contained added tannins, a polyphenolic compound, in order to match the bitter taste of the 600 mg ACN drink, there is likely to have been inhibition of α-

amylase across all test drinks. Thus, the differences observed here are more likely to be related to the other flavonoids contained in the blackcurrant extract.

Concentrations of secondary outcome variables, plasma GIP and GLP-1, were also decreased by the 600 mg ACN treatment throughout the postprandial period (120 min). Medium and lowest dose treatments did not show any effect. The strong inhibition of plasma GIP concentrations suggests that secretory regulation of this incretin is highly sensitive to glucose absorption rates. Intestinal sodium/glucose co-transporter SGLT1 has been shown to be closely related with the expression of GIP and GLP-1 in individual enteroendocrine cells of murine jejunal crypts. Studies in *Sglt1*$^{-/-}$ (knocked out) mice have shown the essential role of SGLT1 as a glucose sensor for gut incretins GIP and GLP-1 secretion (Gorboulev *et al.*, 2012), although intestinal facilitated glucose transporter GLUT2 may also be an important regulator of incretin response (Mace *et al.*, 2012). These findings showing inhibition of GIP and GLP-1 secretion by blackcurrant polyphenols agree with a previous report on inhibited glucose induced SGLT1-mediated incretin secretion by apple polyphenol phlorizin (Moriya *et al.*, 2009), a dihydrochalcone well-known for its SGLT1 inhibitory effects (Smith *et al.*, 1992, Wright *et al.*, 2011). The suppression of plasma GLP-1 concentrations by the highest dose of blackcurrant extract at 90 min paradoxically coincided with the time point where plasma insulin concentrations were increased relative to placebo. Our data support the role of incretins as sensitive biomarkers for the rate of glucose transport into enterocyte, since GIP, GLP-1 and insulin profiles were not in alignment along the postprandial period as it might be expected given the association between insulin and glucose concentrations. GLP-1 is secreted from L cells in the distal small intestine within minutes of nutrient ingestion via neuroendocrine signalling, but the inhibition by the 600 mg ACN treatment occurred around the time that the meal contents would be reaching the part of the small intestine where the secretory cells are located suggesting an inhibition by direct blackcurrant polyphenols contact with the L-cells (Baggio and Drucker, 2007). The inhibition of plasma GLP-1 secretion in the second half of the postprandial period contradicts a previous study of a mixed berry meal consumed with sucrose, showing plasma GLP-1 concentrations increased in the first hour following consumption of the meal (Törrönen *et al.*, 2012c). Opposite findings might mirror differences in study design such as the solid state of the carbohydrate load, and mixed composition (sucrose and starch) of the test meal in the present study. Differences in carbohydrate type and physical state of the test meal might also explain the results in a

previous study showing a return to insulin baseline concentrations following their reference meal (sucrose and water) (Törrönen *et al.*, 2012a), while plasma insulin concentrations in our study remained markedly elevated following control and blackcurrant extract treatments.

Our findings suggest that the consumption of the highest dose of blackcurrant extract caused a slower rate of glucose absorption in the small intestine. Inhibition of digestive enzymes and intestinal glucose transporters (e.g. SGLT1, GLUT2) are the main proposed mechanisms by which blackcurrant polyphenols might act on glycaemic response (Williamson, 2013, Kwon *et al.*, 2007, McDougall, 2008). A third mechanism suggest that as well as glucose anthocyanins can be transported into the enterocyte using intestinal glucose transporters, and competition for this route following an anthocyanin-rich meal might reduce the rate of glucose absorption (Kamiloglu *et al.*, 2015).

Other variables related to lipids, blood pressure, vascular function and oxidative stress were not affected by the 600 mg ACN treatment or any other treatment. Contrary to expectations the insulin-mediated suppression of circulating NEFA concentrations was not different in the current study, our data disagree with a previous study showing an inhibition of the upwards return to baseline in plasma NEFA concentrations around 90-120 min following blackcurrant puree and blackcurrant juice, reflecting insulin concentrations at these times points in comparison to the reference meal (Törrönen *et al.*, 2012a). As for vascular function variables, our results are consistent with previous observations following berry polyphenols using similar methodology (Rodriguez-Mateos *et al.*, 2013), or Laser Doppler imaging with iontophoresis to measure microvascular function (Jin *et al.*, 2011). Possibly a more specific method such as flow-mediated dilatation of the brachial artery by ultrasound is necessary in order to detect an effect of blackcurrant polyphenols on endothelium-dependent vasodilation (Rodriguez-Mateos *et al.*, 2013). Since anthocyanins are rapidly absorbed and cleared, and their plasma concentrations are very low, they are unlikely to have a significant impact on vascular function in the early postprandial period (Rechner *et al.*, 2002). However blackcurrant phenolic acids that peak around 2 h post-ingestion and their colonic metabolites, conjugated phenolic acids, which would be expected to appear in the circulation 4-12 h post-ingestion might be associated with some improvement in endothelial function (Rodriguez-Mateos *et al.*, 2013, Czank *et al.*, 2013). In order to appropriately test vascular function improvement as a primary hypothesis an acute study

of longer duration is required and a larger sample size to allow for inter-individual variability in colonic gut microflora profiles. A decreased glycaemia induced by the highest dose of blackcurrant phenolics might be expected to reduced endothelial oxidative stress and therefore improve vascular function (Del Bo *et al.*, 2015). The deterioration in vascular function variables at 120 min were unaffected by treatment and this may be because the differences in glycaemic response between placebo and highest dose, although statistically significant, were not marked enough to affect vascular function.

Previous data shows that anthocyanins may have an antioxidant effect. However in the present study blackcurrant polyphenols did not influence plasma concentrations of F2-isoprostanes, which are peroxidation products of arachidonic acid arising from reactive oxygen species production and a sensitive marker of cellular oxidative damage. This result suggests that it is unlikely that a reduction of oxidative stress via a direct route has also occurred in the relatively short timeframe for absorption of phenolics into the circulation, although a longer postprandial measurement period might have detected differences at later timepoints. Plasma isoprostanes concentrations increased at 90 min after an OGTT in T2D patients (Sampson *et al.*, 2002), therefore it might be possible that analysis at 120 min in the current study missed an earlier effect.

These results combined provide a unique dataset showing that a highly purified liquid blackcurrant extract (5.4% weight total phenolic content, without fibre and low in other confounding nutrients) not only decreased glucose and insulin concentrations but also inhibits gut incretins GIP and GLP-1 secretion.

The strengths of this study include the adherence to the highest standards of conducting a randomised clinical trial: fully blinded until the final statistical analysis had been completed, randomisation and allocation concealment conducted at a remote site by an independent party and the control treatment fully matched to the intervention drinks for taste, appearance, and nutrient composition. Our results could be applicable to the general population since the study was performed in a broad cross-section of healthy men and postmenopausal women, although for reasons of avoiding the confounding effects of cyclical fluctuations of reproductive hormones on glycaemia and insulinaemia premenopausal women were excluded (Brennan *et al.*, 2009). Furthermore, the highest dose of blackcurrant extract is within physiological levels, since intake of 600 mg of

blackcurrant anthocyanins could be easily achieved in a normal diet by consuming ~100 g of fresh blackcurrant fruit (Rothwell *et al.*, 2013) and the prototype drinks formulated for the study could feasibly be developed into functional drinks and made available to all consumers.

Limitations of the GLU-BERRY study include the addition of tannins as a taste-blinding to the control and lower doses of blackcurrant extract drinks in order to match the bitter/astringent taste of the highest dose of blackcurrant extract. Given that it is impossible to find a bitter/astringent taste additive that is not a bioactive compound there is not an alternative for a replacement for tannins, which are likely to bind to proteins including digestive enzymes. Tannins are complex flavonoid polymers (proanthocyanidins) that have shown *in vitro* inhibitory activity on α-amylase and α-glucosidase (Schafer and Hogger, 2007, Grussu *et al.*, 2011, McDougall *et al.*, 2005), mechanisms which may reduce glucose absortion in gut lumen. Tannins have also been associated with stimulation of the incretin GLP-1 activity and insulin secretion by pancreatic beta cells, mechanisms involved in glucose homeostasis (Blade *et al.*, 2016). Although we observed significant inhibition of glycaemia, insulinaemia and incretin secretion, the true size of the effect may have been larger if the stringent adherence to taste-matching the placebo and lower doses had not necessitated the addition of tannins. The use of a blackcurrant extract rather than a purified blackcurrant anthocyanins source means that it is not possible to attribute with any certainty the effects on glucose homeostasis entirely or even partially to blackcurrant anthocyanins, as there are other polyphenols present in the blackcurrant extract that may have been responsible. Although we did not carry out analysis of other polyphenols in the blackcurrant extract, proanthocyanidins and phenolic acids are most likely to account for the remaining 997 mg (highest dose), 428 mg (medium dose), and 239 mg (lower dose) phenolic content per 200 ml estimated by the Folin-Ciocalteu assay of the drinks.

3.6.2 GLU-APP study

The aim of this study was to show an inhibitory effect on postprandial glycaemia and insulinaemia after the consumption of two different doses of apple polyphenols (approximately 7% phlorizin) contained in no added sugar fruit drinks. It was hypothesised that a dose response effect would be observed following the intake of test drinks immediately before a carbohydrate (glucose, in the form of dextrose monohydrate) load equivalent to an oral glucose tolerance test. A glucose load was used instead of a starch and sucrose meal because of the known inhibitory effects of the apple-specific dihydrochalcone, phlorizin, on SGLT1-mediated transport of glucose, explained below. Results showed no effect of apple extract on circulating concentrations of glucose, insulin, GIP, GLP-1, TAG, or NEFA, or on blood pressure and vascular function. Our data differ from other clinical trials using apple-derived products, like apple juice and apple extract (Johnston *et al.*, 2002, Schulze *et al.*, 2014), which showed a delay in early glucose and insulin response.

Although the apple extract was analysed, the drinks composition analysis has not been provided by the industrial collaborators at the time of writing. It is likely that the same protocol was followed as for GLU-BERRY drinks formulation where tannins were added to the placebo and lower dose to match drinks for astringent/bitter taste. The addition of tannins (proanthocyanidins) to the control (0 mg APP) and lowest dose (300 mg APP) drinks could possibly mask the potential effects of the apple extract treatment on postprandial glycaemia. Since the test meal provided was a liquid load of glucose devoid of starch or sucrose and there were no action of digestive enzymes that could be inhibited, it might be expected that the main effect of apple polyphenols were exerted at the gut luminal interface, by inhibition of glucose transporters (SGLT1 and GLUT2). The apple extract used was rich in flavan-3-ols (~63%) mainly catechin and epicatechin, proanthocyanidins (~30%) and phlorizin (~7%) (**Table 4.3**). Phlorizin is a well-known competitive inhibitor of SGLT1 (Wright *et al.*, 2011) and large polymers such as proanthocyanidins have shown non-specific binding with plasma membrane phospholipids forming complexes that could cover transmembrane proteins (Tarahovsky, 2008) like glucose transporters, hindering their function. Furthermore, covalent and non-covalent interactions are likely to occur between phenolic compounds and proteins (Kroll *et al.*, 2005) impeding the adequate function of the proteins.

However other phenolic compounds present in considerable quantities in GLU-APP test drinks as catechin (39%) and epicatechin (16.5%) when tested in the Caco-2 *in vitro* system did not show an inhibitory total glucose uptake (SGLT1- and GLUT-mediated) (Shimizu, 2000) but did show a significant decrease on GLUT-mediated glucose uptake (Johnston *et al.*, 2005), opposing results may due to concentrations tested and mechanism involved in transporters inhibition.

Previous clinical trials using apple extracts and liquid glucose meal have showed reduced early postprandial glucose response in healthy volunteers, however the phlorizin content in the apple extracts used, 315 mg (Makarova *et al.*, 2015) and 448 mg (Schulze *et al.*, 2014) highly exceed the phlorizin contents in GLU-APP drinks, 21 and 42 mg in the lowest and highest dose, respectively. High concentrations of catechin and epicatechin, 234 and 99 mg in the GLU-APP top dose, did not improve the inhibitory effect as might be expected if compared with Makarova study (Makarova *et al.*, 2015) where 63.5 and 53 mg of catechin and epicatechin were contained in the extract supplied. Alternatively a human trial using clear and apple juices containing small amounts of phlorizin, 12 and 26 mg, respectively, and a load of sucrose, fructose and glucose showed a significant effect in early glucose response (Johnston *et al.*, 2002), although the complete phenolic profile of the apple juices were not provided, apple juice is estimated to contained significant amounts of proanthocyanidins (Rothwell *et al.*, 2013), which are likely to have contributed to the significant effects on glucose uptake by inhibiting digestive enzymes and limiting glucose availability in the gut lumen. These results suggest that the concentrations of phlorizin contained in GLU-APP drinks, expected to be the major responsible of decreased glucose uptake, were not sufficient to show an inhibitory effect on glucose response and that the inhibition of digestive enzymes may be play a central role in the decreasing of postprandial hyperglycaemia.

Limitations in the GLU-APP study might be the use of a liquid test meal (glucose + water) in contrast to a solid, mixed-carbohydrate meal as used in the GLU-BERRY protocol. Although the glucose meal was chosen in order to induce a higher postprandial glycaemia, and because the phlorizin in the apple extract was expected to be the most bioactive component in directly inhibited SGLT1 transporter, administering a glucose meal instead of a starch/sucrose meal removed the digestive enzyme (α-amylase and α-glucosidase) component of the total inhibitory effect (Barbosa *et al.*, 2010). Moreover the probable addition of tannins to the placebo and lowest dose drinks

might have equalled the proanthocyanidins contained in the highest dose therefore diminished the difference in glucose uptake in gut lumen between treatments and it subsequent effects. Alternatively, the doses chosen, equivalent to 150 and 300 g of whole raw dessert apples (Rothwell *et al.*, 2013) may have been too low to inhibit postprandial glycaemia and a higher dose may have been more efficacious. The doses tested 300 and 600 mg of polyphenols (mainly flavonoids) agreed with the average daily intake in Mediterranean countries as France, Italy, Spain and are even lower than estimated intakes in Northern European countries as UK, Netherlands and Denmark (Zamora-Ros *et al.*, 2015); therefore consumption of 600 mg of polyphenols not exclusively derived from apple might be a daily goal that is easy to achieve.

The use of a liquid load of pure glucose does not represent a typical real life meal and therefore the use of a solid meal containing different type of carbohydrates and other macronutrients as protein and fat represent a closer approach to daily life. Since the doses of apple extract used have had no effect on glycaemia response after the glucose meal and apple extract was 7% phlorizin, our results questions the relative importance of SGLT1 inhibition *in vivo* relative to the potential inhibition of digestive enzyme activity by polyphenols if disaccharides or polysaccharides are included in the test meal. Previous studies suggests that phlorizin, as inhibitor of SGLT1, need to be in greater concentrations if only glucose is provided as meal. In addition the used of disaccharides and/or polysaccharides might allow to see a positive effect with small amounts of phlorizin but a significant quantity of polyphenols contained in extracts.

3.6.3 GLU-FRU study

In this clinical trial we showed an inhibitory effect on postprandial glycaemia and insulinaemia by both treatments; apple polyphenol and the combination of apple polyphenols and blackcurrant anthocyanins, with the combination of both extracts having a more potent effect. The mixed extract drink treatment (AE+BE) reduced by 73% the early glucose response (iAUC 0-30 min) compared to a 63% reduction with the apple extract only treatment (AE). Results from different trials testing acute and chronic hypoglycaemic effect of acarbose, an α-glucosidase inhibitor used in the treatment of T2D, have shown a decreased postprandial glycaemia (AUC) between 12 and 48% (Joshi *et al.*, 2015, Nawawi *et al.*, 2000). Although we did not test chronic effects, given the acute effects showed in our clinical trials it seems likely that the fruit extracts could act in a similar manner than acarbose.

A similar response was found for the gut incretin, GIP; an overall effect was showed after consumption of both treatments with a stronger inhibition following the AE+BE treatment. As might be expected the insulin-mediated postprandial inhibition of plasma NEFA concentrations mirrors the insulin response across treatments, with higher NEFA concentrations following the consumption of AE+BE drink compared to AE and CON. Plasma TAG concentrations, blood pressure and vascular function DVP-SI did not differ between treatments. However, vascular function DVP-RI was decreased over the study period (2 h) by the AE treatment indicating a reduction in systematic arterial resistance.

To our knowledge this is the first study testing the combination of apple extract and blackcurrant extract in a randomised controlled trial. Previously we showed in the GLU-BERRY study that a drink containing 600 mg of blackcurrant anthocyanins (1600 mg total phenolics) inhibit plasma glucose and insulin early response. Results in GLU-FRU study clearly show an increased overall effect on the glucose homeostasis mechanism along the postprandial period by combining 600 mg of apple polyphenols and 600 mg of blackcurrant anthocyanins (1200 mg total blackcurrant polyphenols) in a test drink.

Plasma glucose concentrations were reduced following the AE and AE+BE treatments in the early stage as measured by iAUC (0-30 min). Our results agree with previous reports of clinical trials where consumption of cloudy apple juice and apple extracts showed a significant reduction in plasma glucose concentrations in early postprandial

response (Johnston *et al.*, 2002, Schulze *et al.*, 2014, Makarova *et al.*, 2015). The apple extract used in GLU-FRU study was rich in phlorizin (8.4%), giving a total of 100 and 50 mg in the AE and AE+BE drinks, respectively. In comparison, the apple extract used in GLU-APP although similar in phlorizin content (7%) was lower in total dihydrochalcones, hydroxycinnamic acids and proanthocyanidins. In previous reports a cloudy apple juice containing 26 mg of phlorizin, 25 g glucose and 30.7 g fructose significantly lowered plasma glucose in human volunteers compared to a control drink, as demonstrated by a lower glucose iAUC from 0-30 and 30-90 mins (Johnston *et al.*, 2002). Furthermore, a dose of 2.8 g of an apple extract that appeared to be derived from apple pomace, containing a very large dose (448 mg) of phlorizin, reduced plasma glucose iAUC between baseline and 15, 30 and 45 mins in healthy human subjects following a 75 g glucose load (Schulze *et al.*, 2014), and 25 g of unripe apple extract, derived from apple pomace, containing 315 mg of phlorizin reduced postprandial glucose response in healthy volunteers following by a 50 g glucose load (Makarova *et al.*, 2015). However the magnitude of the inhibition found in our study was clearly greater than previous reports, which might be explained by differences in the carbohydrate load, i.e. the solid state of the meal allowing the inhibitory effect more time to take effect, and the mixed carbohydrate composition, starch and sucrose, in the present study versus a liquid load of glucose, fructose, and sucrose in the studies reported by Schulze *et al.* and Makarova *et al.*, and glucose only in the GLU-APP study. *In vitro* studies have shown inhibitory effects on the activity of digestive enzymes α-amylase and α-glucosidases (maltase and sucrase) by flesh and peel apple extracts (Barbosa *et al.*, 2010), flavonol quercetin (Tadera *et al.*, 2006), proanthocyanidins (Grussu *et al.*, 2011) and other apple polyphenols compounds as chlorogenic acids (Hanhineva *et al.*, 2010).

The apple extract used here was rich in phlorizin as well as in proanthocyanidins and chlorogenic acid in addition small amounts of other characteristic polyphenols as monomeric flavan-3-ols and flavonols were contained. In addition to potential polyphenol-induced delayed digestive breakdown of carbohydrates, inhibition of glucose transporters (SGLT1 and GLUT2) by apple extracts and phlorizin (Manzano and Williamson, 2010, Schulze *et al.*, 2014) as well as quercetin glycosides (Ader *et al.*, 2001) as showed in *in vitro* systems, may explain the marked effect of postprandial glucose and insulin response. SGLT1 inhibition presents a mechanism by which

phlorizin-containing apple extracts and juice may lower the postprandial glucose curve. However, the previously mentioned studies in humans have utilised extracts or juices that contain additional polyphenols from apple. Therefore, it is unlikely that these effects are played out entirely by phlorizin. In a study using mice everted jejunal rings pure phlorizin (4.1μM) proved to be less effective in inhibiting sugar absorption when compared with phlorizin contained in an apple extract (3.2 μM) (Schulze *et al.*, 2014). Although phlorizin was the most effective inhibitor, other polyphenols contained in the apple extract showed inhibitory effects in lower degree.

The polymeric proanthocyanidin, component of apple polyphenols estimated using Phenol Explorer (Rothwell *et al.*, 2013) to constitute 300 and 600 mg of the AE+BE and AE drinks respectively, and consisting of monomer and dimers (~18%), oligomers (3-10 mers) (~52%) and polymers (>10 mers) (30%) has previously shown inhibition of glucose and insulin at 10 and 30 min at doses of 95 and 285 mg when consumed together with a high carbohydrate meal (Sapwarobol *et al.*, 2012). *In vitro* enzymatic studies have suggested non-specific inhibition of α-amylase by direct contact between proanthocyanidins (PA) and the enzyme (McDougall *et al.*, 2005). Interactions between polyphenols might show an additive effect when PA are combined with other phenolic compounds, such as anthocyanins (Akkarachiyasit *et al.*, 2011) or flavonols and flavones (Lo Piparo *et al.*, 2008) which also inhibit the enzyme by a different mechanism as interaction with the active site. Or a synergistic effect when α-amylase inhibitors are in presence of α-glucosidase inhibitors as chlorogenic acid, a hydroxycinnamic acid (McDougall, 2008) or cyanidin-3-glucoside, an anthocyanin (Adisakwattana *et al.*, 2004).

Other human studies showing inhibition of glucose and/or insulin using apple polyphenols have used higher quantities of individual polyphenols than those in the current AE and AE+BE drinks. For example one gram of chlorogenic acid (van Dijk *et al.*, 2009) vs 32 and 16 mg in AE and AE+BE drinks, and 400 mg of quercetin vs 16 and 8 mg in AE and AE+BE drinks (Hussain *et al.*, 2012) which made the current concentrations of these individual polyphenols too low to play a highly significant effect on glucose homeostasis by themselves. However, the present data further confirm the inhibitory actions of polyphenol-rich apple and anthocyanin-rich blackcurrant extracts in modulating the postprandial glucose response, suggesting that additive

and/or synergistic effects of the different polyphenols present in the extracts might explain the overall results on glucose homeostasis.

Plasma insulin concentrations follow the glucose response, with a decreased concentration following consumption of AE and AE+BE treatments up to 45 min, compared to control and a marked effect following the mixed extract treatment. Since insulin concentrations are not only subject to pancreatic secretion rates, but also to the rate of hepatic extraction, we also analysed C-peptide concentrations, which is a polypeptide originated in the β-cells and is removed from the proinsulin molecule to produce insulin. Is secreted in equal concentrations as insulin into portal circulation from where a percentage of insulin is retained by liver but no C-peptide is extracted and reaches plasma circulation representing a better marker of glucose induced β-cell secretion. Concentrations and plasma kinetics of C-peptide therefore indicate rate of insulin secretion more accurately than plasma insulin concentrations (Hoekstra *et al.*, 1982). Plasma concentrations of C-peptide were reduced following the AE and AE+BE treatments up to 60 min, with a pronounced inhibition by AE+BE treatment.

In concordance with the data on insulin secretion, plasma concentrations of the incretin, GIP, were also decreased by treatments AE and AE+BE throughout the 2 hours postprandial period. There was a marked effect in early response following the AE+BE as measured by iAUC 0-30 min. As observed in the GLU-BERRY study the strong inhibition of plasma GIP concentrations suggests that secretory regulation of this incretin is highly sensitive to glucose absorption rates. Intestinal sodium/glucose co-transporter SGLT1 has been shown to be physically and physiologically related with the expression of GIP, and SGLT1 may act as a glucose sensor for GIP secretion (Gorboulev *et al.*, 2012). GIP is secreted in the proximal section of small intestine by K-cells only minutes after carbohydrate and fat intake (Baggio and Drucker, 2007). GIP secretion was decreased up to 75 min of the postprandial period corresponding to the time that meal content should take to transit along the upper intestine (duodenum and jejunum); it is possible that decreased stimulation of GIP secretion results from direct inhibitory phlorizin contact with K-cells (Smith *et al.*, 1992, Wright *et al.*, 2011), as well as synergistic inhibition by blackcurrant polyphenols in the case of AE+BE, which contained half the dose of phlorizin and showed a greater effect on GIP secretions than AE. Our results agree with a previous report of inhibition of glucose-induced SGLT1-mediated incretin secretion by phlorizin in mice (Moriya *et al.*, 2009), although

experiments using a rat model suggests GLUT2 might also play a role as glucose sensor for glucose induced incretin secretion (Mace *et al.*, 2012) and this presents an additional mechanism whereby polyphenols such as phloretin, a GLUT2 inhibitor, may influence gut hormone secretion. Although phloretin was only present in low amounts in this apple extract, phlorizin could be rapidly cleaved by the enzyme lactase phlorizin hydrolase (LPH) to release the aglycone phloretin.

Plasma NEFA concentrations followed the expected insulin-mediated suppression following treatment AE and AE+BE, concentrations were higher following AE and AE+BE treatments compared to CON, with a marked effect for AE+BE mirroring plasma insulin and GIP concentrations, effect was observed throughout the postprandial period. There are no previous studies showing effects on non-esterified fatty acids concentrations by apple extracts or apple juices, but our results suggests an evident influence of both treatments on circulating NEFA concentrations after ingestion of apple and blackcurrant extracts and a high carbohydrate meal, as a side-effect of reduced insulin secretion, and ultimately inhibition of intestinal glucose absorption. Other variables related to plasma lipids and blood pressure were not affected by any treatment.

This study hypothesised that an improvement in vascular function measured as digital volume pulse stiffness and reflection index would be observed following the AE and AE+BE treatment, with a more marked effect after AE+BE treatment. Our results show no effect on DVP-SI (mainly reflecting stiffness of larger arteries) across treatments; however the increase from baseline for DVP-RI (indicating vascular resistance of small to medium-sized arteries) was suppressed following AE throughout the postprandial period. There was a tendency for decreased values following the AE+BE treatment but the difference did not reach statistical significance. This result is an agreement with GLU-BERRY study where the same amount of anthocyanins (600 mg) did not show an improvement in vascular function. The additive effects of apple and blackcurrant extract observed in glucose homeostasis variables did not have a highly significant effect on vascular function, suggesting that the reductions in pulse wave reflection were directly related to the post-absorptive effects of absorbed polyphenols on the vascular wall rather than an indirect effect via reduced postprandial glycaemia-induced oxidative stress.

Our results are in agreement with previous reports showing an improvement in vascular function measured by flow mediated dilation of the brachial artery, a more specific methodology, following a high-quercetin and high-epicatechin apple treatment (Bondonno *et al.*, 2012). Our data also agree with human studies supporting the putative mechanisms of apple polyphenols on endothelial function improvement, mainly by increasing the bioavailability and bioactivity of nitric oxide (NO) in circulating plasma, a main regulator of vasodilation (Bondonno *et al.*, 2012, Hollands *et al.*, 2013, Gasper *et al.*, 2014). Several *in vitro* studies have shown the potential pathways involved including activation of endothelial NO synthase (eNOS) by quercetin and quercetin metabolites (Shen *et al.*, 2012), increase eNOS expression by catechins compounds (Appeldoorn *et al.*, 2009), inhibition of NADPH oxidase by *Orto*-methylated flavan-3-ols and chlorogenic acids (Steffen *et al.*, 2008) causing a decrease in reactive oxygen superoxide formation. A human study showed that ~3% of an ingested dose of chlorogenic acids were absorbed in the 0-2 h period after intake (Stalmach *et al.*, 2009) therefore it is probable that chlorogenic acids contained in the test drink and absorbed within the 2 h postprandial period may have contributed to increase NO bioavailability. Although the polyphenols showing a significant effect in *in vitro* studies were in our apple extract, quantities contained in drinks are too low in comparison with effective doses *in vitro*; an additive effect might explain the vasculo-protective effect of AE treatment. An explanation of possible cause-effect relationships need to be further explored, since differing rates of absorption and metabolic fates of the combined polyphenols may have a relation with the observed improvement in vascular function as has been suggested by a previous report (Rodriguez-Mateos *et al.*, 2013).

Strengths of this study, as for GLU-BERRY and GLU-APP, include the robust study design and the use of highly purified fruit extracts removing the confounding factor of fruit fibre and ensuring the homogenous polyphenol content in the drinks, eliminating the variability in polyphenol content on fruits due to species and cultivars and genetic, agricultural and environmental factors as well as storage and processing conditions (de Pascual-Teresa *et al.*, 2007, Tabart *et al.* 2006, Basu *et al.*, 2014).

The dose of blackcurrant extract used is within achievable levels, since intake of 600 mg of blackcurrant anthocyanins could be easily achieved in a normal diet by consuming ~100 g of fresh blackcurrant fruit. However, intake of 600 mg of apple polyphenols would require consumption of ~300 g of fresh dessert apple fruit which is

plausible but challenging to have in one sitting. Furthermore, the apple extract used was especially high in phlorizin, which is more concentrated in apple stem (pedicel), seeds and skin (exocarp). Treatment AE+BE contained ~50 mg of phlorizin which represents the amount contained in 18 medium dessert unpeeled apples (1800g) (Rothwell *et al.*, 2013).

The drinks were matched for their content on apple polyphenols and blackcurrant anthocyanins rather than blackcurrant polyphenols, which lead to a higher amount of total phenolics content on the AE+BE than in the AE drink, although due to the diverse nature and functional properties of polyphenols then it is arguable that this is not a hindrance to interpretation of the results. The combination of extracts and the potential presence of un-identified polyphenols contained within them, makes it difficult to attribute the effects on glucose homeostasis and vascular function entirely or even partially to blackcurrant anthocyanins or specific apple phenolic acids or flavonoids, as there are other polyphenols present in the extracts that may have been responsible. Intake of 1200 mg of apple polyphenols as used in AE treatment is achievably in a normal diet by consuming ~600 g of fresh apple fruit which is unrealistic unless pureed and concentrated. Further studies on the dose-response relationship are needed to identify the minimum effective dose required in order to achieve the same clinical effects.

3.6.4 Conclusion

In conclusion, the ingestion of blackcurrant and apple polyphenol-rich extracts decreased the plasma levels of glucose following starch and sucrose containing meals (but not following a glucose meal in the case of apple), and inhibited the secretion of insulin, GIP and to a smaller extent GLP-1. Although blackcurrants are likely to be consumed only sporadically in the general population and its availability is geographically limited, other berries more widely consumed like redcurrants, strawberries, grapes, raspberries, blackberries, blueberries, bilberries and cranberries contain different profiles of anthocyanins and proanthocyanidins. The consumption of a large dose of apple polyphenols also decreased circulating NEFA concentrations and improved markers of vascular function within the 2 h postprandial timeframe following a starch/sucrose meal. A mixture of apple and blackcurrant polyphenols attenuated in a more marked way the glucose, insulin, GIP and NEFA responses than apple or

blackcurrant polyphenols did on their own. Intakes of apple polyphenols in equivalent doses to that used in our study is challenging to achieve by consumption of fresh fruit, but since apple is consumed frequently in the general population and is extensively available, then the cumulative impact of daily small inhibitory effects on postprandial glycaemia could potentially still have an impact on health in the long term. Therefore regular consumption of berries, apples or a combination of both as main meals or with snack foods may help lowers postprandial glycaemia and insulinaemia. A more gradual and sustained insulinaemic response could potentially increase satiety during intermeal intervals (Blaak *et al.*, 2012) and a lowered postprandial glycaemia is likely to protect the optimum functionality of pancreatic beta cells and may help to reduce the vascular burden of glucose-induced oxidative stress and endothelial dysfunction (Williams *et al.*, 1998, Ceriello *et al.*, 2008) associated with the development of atherosclerosis. See **Table 3.15** for a summary of effects on glucose homeostasis and vascular function showed by GLU-BERRY and GLU-FRU studies.

Table 3.15 Summary table of GLU-BERRY and GLU-FRU studies outcomes

Outcome	**GLU-BERRY** (600 mg ACN)	**GLU-FRU** (600 mg AE + 600 mg ACN)	**GLU-FRU** (1200 mg AE)
Glucose	↓ AOB 0-30 min	↓ iAUC 0-120 min	↓ iAUC 0-30 min
Insulin	↓ AOB 0-30 min	↓ iAUC 0-120 min	↓ iAUC 0-120 min
C-peptide	NA	↓ iAUC 0-120 min	↓ iAUC 0-30 min
GIP	↓ AOB 0-120 min	↓ iAUC 0-120 min	↓ iAUC 0-120 min
NEFA	NS	↑ iAUC 0-120 min	NS
DVP-RI	NS	NS	↓ Δ 60, 90, 120 min

ACN, anthocyanin; AE, apple extract; AOB, area over baseline; iAUC, incremental area under the curve; NA, not analysed; GIP, glucose-dependent insulinotropic; polypeptide NS, no statistically significant; NEFA, non-esterified fatty acids; DVP-RI, digital volume pulse – reflection index.

Given the concentrations of polyphenols used in the RCTs, incorporation of individual or blended blackcurrant and apple extracts into novel food or drink products, such as no added sugar fruit drinks, cereal bars, wholegrain crackers, breads and pasta, offers a potential avenue of functional food development that could target consumers (healthy or with T2D) who are interested in controlling their blood glucose concentrations. However, there may be significant technical challenges for the food and drink industry in formulating products with the desired characteristics in terms of physical and chemical stability (Woodward *et al.*, 2011), bioavailability (Gonzales *et al.*, 2015) and palatability (Drewnowski and Gomez-Carneros, 2000). Most phenolic compounds are known and even prized (particularly in the case of tea, coffee and wine) for their astringency and bitterness, but these properties are not always desirable in other foods so additional ingredients can sometimes be added to overcome these sensory challenges, for example, gums and pectins (Laaksonen *et al.*, 2015). However, it is possible that addition of polysaccharides would enable the polyphenols to bind to these instead of the digestive enzymes, thus diminishing their glycaemia-lowering effects, and considerable efforts would be required by the food industry to develop acceptable novel products fortified with fruit extracts.

The scientific evidence presented here supports current dietary guidelines that consumption of meals rich in fruits and vegetables and wholegrains may help prevent T2D and CVD, and suggests one of many routes by which cardio-metabolic protection can occur. Further mechanistic investigations into the polyphenol subclasses and/or individual polyphenols that are responsible for these clinical effects would strengthen current knowledge in the area. Elucidation of mechanisms involving glucose transporters expressed in *in vitro* systems is the next step in this research project, where blackcurrant and apple extracts used in RCTs were tested for their inhibitory effect on intestinal glucose uptake.

Chapter 4

Acute effects of anthocyanin-rich blackcurrant extract and polyphenol-rich apple extracts on glucose transport in human intestinal cell line Caco-2/TC7 and *Xenopus laevis* oocyte systems.

4.1 Introduction

Consumption of foods with high content of polyphenols has been associated with reduced risk for type 2 diabetes. Epidemiological studies have shown an association between flavonoids and phenolics acids and lower risk of T2D (Zamora-Ros, 2013, Wedick *et al.*, 2012) and randomised controlled trials have shown inhibition of postprandial glycaemia after consumption of polyphenol-rich meals or drinks (Törrönen *et al.*, 2012c, Törrönen *et al.*, 2012a, Schulze *et al.*, 2014, Johnston *et al.*, 2002). In our clinical trials, we have shown a decrease in postprandial glycaemia after consumption of drinks containing an anthocyanin-rich blackcurrant extract and a proanthocyanidin- and chlorogenic acid-rich apple extract. Potential mechanisms for hypoglycaemic effect of polyphenols include inhibition of digestive enzymes, decrease of intestinal glucose absorption, and stimulation of insulin secretion and protection of pancreatic β-cells.

During digestion of carbohydrates, digestive enzymes, α-amylases and α-glucosidases, play a central role degrading polysaccharides and oligosaccharides into shorter-chain oligosaccharides, disaccharides and ultimately, monosaccharides. Certain polyphenols such as anthocyanins, flavonols, flavan-3-ols, proanthocyanidins and phenolic acids may inhibit α-amylases and α-glucosidases activity (Adisakwattana *et al.*, 2004, McDougall *et al.*, 2005, Tadera *et al.*, 2006, Adisakwattana *et al.*, 2009, Akkarachiyasit *et al.*, 2010, Pereira *et al.*, 2011, McDougall, 2008), slowing the accumulation of free glucose in the small intestine. Once free glucose is released, it is ready for transporter-mediated uptake into enterocytes; however *in vitro* experiments have shown that certain polyphenols such as anthocyanins, flavan-3-ols, flavonols and dihydrochalcones may directly inhibit intestinal glucose transporters SGLT1 and GLUT2 (Johnston *et al.*, 2005, Kwon *et al.*, 2007, Manzano and Williamson, 2010, Alzaid *et al.*, 2013, Schulze *et al.*, 2014), slowing the rate of glucose influx into enterocytes and subsequent efflux to the blood stream. Although there is a considerable number of studies showing polyphenol effects on intestinal glucose transporters, results should not be generalised. Effective concentrations and mechanisms of action vary widely between polyphenols, moreover concentrations tested have not always agreed with *in vivo* conditions limiting biologically significant conclusions.

Therefore the aim of the present project was to identify the mechanisms of glucose uptake inhibition by physiological concentrations of blackcurrant and apple extracts

used in our randomised clinical trials, on intestinal sugar transport using Caco-2/TC-7 cells and *Xenopus laevis* oocytes systems.

4.2 Hypothesis

Anthocyanin-rich blackcurrant extract, individual major blackcurrant anthocyanins, and polyphenol-rich apple extracts inhibit glucose uptake in a dose-responsive way in human intestinal cell line Caco-2/TC7 and *Xenopus laevis* oocytes injected to express sodium-dependent glucose transporter 1 (SGLT1).

A secondary hypothesis was that, should the blackcurrant extract be shown to inhibit glucose uptake in cell culture, this was at least partly mediated by blackcurrant anthocyanins.

4.3 Methodology

In vitro experiments were completed under supervision of Dr Christopher P Corpe and with the assistance of postgraduate research students, Dr Patrick O'Brien, Dr Hoi Man Cheung and Diana C Munoz Sandoval.

4.3.1. Polyphenol-rich fruit extract profiles

Three different polyphenol-rich fruit extracts used in randomised clinical trials (Chapter 3) were tested in the Caco-2/TC7 and *Xenopus* oocyte systems. Details on extracts composition and concentrations used in the *in vitro* assays are shown below.

4.3.1.1 Anthocyanin-rich blackcurrant extract (BE)

The anthocyanin-rich blackcurrant extract (BE) (BerryPharma® by Iprona AG, Lana, Italy) used in GLU-BERRY and GLU-FRU studies contained 2822 mg total anthocyanins per 100 ml (5775 mg total polyphenols per 100 ml), analysed by RSSL The Lord Zuckerman Research Centre (Reading, UK) (**Table 4.1**).

Table 4.1 Anthocyanin-rich blackcurrant extract (BE) profile

Compound	**Concentration** (mg/100 ml)
Total anthocyanins	2822
Cyanidin-3-glucoside	156
Cyanidin-3-rutinoside	986
Delphinidin-3-glucoside	359
Delphinidin-3-rutinoside	1226
Total polyphenols	5775
Total carbohydrates	2300
Glucose	100
Fructose	100

All values were derived from HPLC analysis of raw extract

Individual anthocyanins and anthocyanidins in BE

The four main anthocyanins present were glycoside compounds of delphinidin and cyanidin. Is has been proposed that anthocyanin glycosides may be cleaved by lactase phlorizin hydrolase inserted in enterocytes provoking the release of free aglycones of the parental compounds, in this case delphinidin and cyanidin. Therefore the four main anthocyanins present in the blackcurrant extract and the two parental aglycones (anthocyanidins) were tested (**Table 4.2**) as well as combination of the four glycosides and combination of the two aglycones.

Table 4.2 Anthocyanins and anthocyanidins tested in Caco-2 cell system

Individual compound	**Supplier details**	**Solvent used**[1]
Cyanidin-3-glucoside (Kuromanin chloride)	Santa Cruz Biotechnology, cat no.sc-235457	DMSO
Cyanidin-3-rutinoside (Keracyanin chloride)	Santa Cruz Biotechnology, cat no. sc-228384	DMSO
Cyanidin (Cyanidin chloride)	Santa Cruz Biotechnology, cat no. sc-202559	Methanol
Delphinidin-3-glucoside (Delphinidin 3-β-D-Glucoside)	Santa Cruz Biotechnology, cat no. sc-207518	Ethanol
Delphinidin-3-rutinoside (Delphinidin-3-rutinosidchlorid)	LGC Limited, PLB-80735	Methanol
Delphinidin (Delphinidin chloride)	Cayman Chemical Company cat no. 11012	DMSO

[1]Solvent stated by the supplier

4.3.1.2 Polyphenol-rich apple extract (AE1)

Polyphenol-rich apple extract (AE1) used in GLU-APP study (JF-NATURAL, Tianjin Jianfeng Natural Product R&D Co., Ltd, China) contained 73 g of total polyphenols per 100 g of extract, analysed by RSSL The Lord Zuckerman Research Centre (Reading, UK) (**Table 4.3**).

Table 4.3 Polyphenol-rich apple extract profile (AE1)

Polyphenol	**Concentration** (mg/100 g)
Dihydrochalcones	
Phloretin	110
Phlorizin	4992
Flavonols	
Quercetin	103
Hydroxycinnamic acids	
Gallic acid	< 30
Flavan-3-ols (monomers)	
Catechin	28479
Epicatechin	12010
Epigallocatechin	5467
Gallocatechin	< 1
Epigallocatechin gallate	< 10
Gallocatechin gallate	< 100
Epicatechin gallate	182
Proanthocyanidins	21839
Total polyphenols	73000

All values were derived from HPLC analysis of raw extract

4.3.1.3 Polyphenol-rich apple extract (AE2)

The polyphenol-rich apple extract (AE2) (Appl'In^{TM} by DIANA FOOD, Antrain, France) used in GLU-FRU study contained 67% phenolics as analysed by DIANA FOOD and confirmed by author using Folin-Ciocalteu method (Singleton and Rossi, 1965) (**Table 4.4**).

Table 4.4 Polyphenol-rich apple extract profile (AE2)

Compound	**Concentration** (mg/100 g)
Dihydrochalcones	10050
Phlorizin	5360
Flavonols	3350
Quercetin	670
Hydroxycinnamic acids	2680
Chlorogenic acid	2010
Flavanols	10720
Epicatechin	134
Procyanidins	10586
Proanthocyanidins	40200
Total polyphenols	67000
Total carbohydrates	8500
Glucose	1300
Fructose	3000

All values were derived from HPLC analysis of raw extract

4.3.1.4 Estimation of physiological concentrations of BE, AE1 and AE2

Estimation of physiological concentrations of blackcurrant polyphenols, blackcurrant anthocyanins and apple polyphenols present in gut lumen after ingestion of test drinks in clinical trials were based on the milligrams of total polyphenols or anthocyanins present in the test drinks, the volume of test drinks (0.2 L) and a conservative estimate

of the maximum volume of gastrointestinal fluid within which drinks would be diluted after ingestion (up to 2 L) with a solid meal (Kong and Singh, 2008; Schiller *et al.*, 2005).

Concentration of blackcurrant polyphenols and blackcurrant anthocyanins present in test drinks used in GLU-BERRY were described in **Table 3.1**, test drinks contained 460, 810 and 1596 mg of polyphenols. The total volume where polyphenols dissolved was predicted to be 2.2 L. Therefore estimated physiological concentrations of blackcurrant polyphenols in gut lumen after consumption of test drinks were 0.21, 0.34 and 0.73 mg/ml. For practical reasons concentrations *in vitro* were tested as 0.2, 0.4 and 0.8 mg/ml, additional lower and higher concentrations were also tested.

Concentrations of individual blackcurrant anthocyanins tested corresponded with estimated physiological concentrations of individual compounds present in gut lumen after consumption of the top dose drink; 600 mg ACN for details in calculation see **Table 4.5**.

Table 4.5 Estimated physiological concentrations of anthocyanins and anthocyanidins tested in Caco-2 cells

Individual compounds in blackcurrant extract	**ACNs in top dose drink** (mg)	**Concentration in gut lumen** (mg/ml)	**MW**	**Concentration in gut lumen** (mM)
Cyanidin-3-glucoside[1]	33	0.015	485	0.03
Cyanidin-3-rutinoside[1]	209	0.095	631	0.15
Delphinidin-3-glucoside[1]	76	0.035	501	0.07
Delphinidin-3-rutinoside[1]	260	0.118	647	0.18
Total anthocyanins	599	0.272	557[3]	0.47
Cyanidin[2]	242	0.110	323	0.18[4]
Delphinidin[2]	336	0.153	339	0.25[4]

ACNs, anthocyanins/anthocyanidins; MW, molecular weight; mM, millimolar.
[1] Concentrations estimated from the analysis of the raw extract and not from drinks analysis.
[2] Values estimated from glycosides concentrations in raw extract.
[3] Averaged molecular weight of main anthocyanins.
[4] Added up of their correspondent glycosides.

Concentrations of apple polyphenols present in test drinks used in GLU-APP and GLU-FRU studies were described in section 3.4.2 and **Table 3.2**, respectively. Test drinks contained 300, 600 and 1200 mg of apple polyphenols, the total volume where apple polyphenols dissolved was predicted to be 2.2 L, therefore estimated physiological concentrations of apple polyphenols in gut lumen after consumption of 300, 600 and 1200 mg of apple polyphenols were 0.14, 0.27 and 0.54 mg/ml, respectively. For practical reasons concentrations *in vitro* were tested as 0.15, 0.3 and 0.5 mg/ml, respectively. Additional lower and higher concentrations were also tested in the *in vitro* systems.

4.3.2 Human intestinal Caco-2/TC7 cells system

4.3.2.1Cell culture conditions

The human intestinal Caco-2 cell line TC7 subclone was obtained from Dr Paul Sharp/Christopher Corpe's laboratory at KCL. Cells were grown, maintained and subcultured following procedure previously reported (Kwon *et al.*, 2007). Caco-2/TC7 cells were seeded in T-25 cell culture flasks (Corning®, Sigma-Aldrich), cells were grown in high-D-glucose (25 mM) with glutamine Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, UK), containing 10% fetal bovine serum (FBS; Sigma-Aldrich), 50 units penicillin and 50 µg streptomycin (Pen/Strep; Sigma-Aldrich), 0.1 mM MEM non-essential amino acids (Life Technologies, UK), additional 2 mM L-Glutamine (Life Technologies, UK) and 5 µg/ml Plasmocin (InvivoGen, USA), referred as complete media in the following sections. Cells were cultured at 37^{o}C in a humidified incubator (BIOHIT, HealthCare, UK) in a 5% CO_2-95% air atmosphere. Complete medium was changed every other day and cells were split once a week when 80 % confluence was reached. For detaching cells the monolayer in T25 flask was washed twice with 4 ml of sterilised 37 ^{o} C PBS, subsequently 1.5 ml of TrypLE^{TM} Select (Life Technologies, UK) were added and flask was incubated 15 min at 37 ^{o} C. After detaching the cells, a suspension was made adding 8.5 ml of complete medium and centrifuged at 1000 rpm for 5 min at 23 ^{o} C. Supernatant medium was discarded and the cell pellet was re-suspended in 10 ml of complete medium. For counting cells 15 µl of the cell suspension was loaded on the haemocytometer, a cover slide was place before in order to add the suspension by capillarity. For passaging cells were seeded at 6000 cells/cm^2 density in T25 flasks and for experiments cells were seeded at 8,000 cells/cm^2

density in 24-well dishes (NUNC, DK) and grown for 21 days, cells were used between passages 45-53 in all experiments.

4.3.2.2 Glucose uptake assays

Conditions for glucose uptake assays were previously established in the laboratory during the doctoral program of Dr Patrick O'Brien (O'Brien, 2015). For glucose transport assays uptake media contained 10 mM D-glucose or 10 mM L-glucose and 0.1 µCi/ml Glucose D-[^{14}C(U)] or 0.1 µCi/ml Glucose L-[1-^{14}C] as tracer (**Table 4.6**). Krebs buffer solution (KBS) containing 30 mM HEPES, 130 mM NaCl, 4 mM KH_2PO_4, 1 mM $MgSO_4 \cdot 7H_20$, 1 mM $CaCl_2$, dH_2O and adjusted pH 7.4 was used as wash media, for sodium-free conditions NaCl was replaced with KCl. Sodium-containing and sodium-free KBS supplemented with 0.2% bovine serum albumin (BSA) was used as pre-incubation and uptake media. Times for pre-incubation and uptake in all experiments, unless specified, were 15 and 10 minutes, respectively. Throughout assays pre-incubation started by aspirating complete media, washing cells with KBS and adding 500 µl of pre-incubation media. Uptake was initiated by removing pre-incubation media and adding 500 µl of uptake media and terminated by aspirating uptake media and washing cells three times with 500 µl of ice cold 1 M phosphate buffer solution (PBS). Subsequently 500 µl of 0.2% sodium dodecyl sulphate (SDS) were added to each well and plates were incubated one hour at 37 °C for cellular lysis. After incubation aliquots of 200 µl of cell lysate were added to 5 mL of Ecoscint A Scintillation Cocktail (National Diagnostic) and radioactivity count was measured on a Liquid Scintillation Counter (Beckman Coulter, Inc. LS6500). Calculations for glucose uptake were made taking into account the specific activity of uptake media, glucose uptake was corrected for simple diffusion of L-glucose.

4.3.2.2.1 Inhibitors of glucose transporters

To ensure the Caco-2/TC7 system was functioning as expected specifics inhibitors of sugar transporters were tested. Glucose uptake experiments were performed under sodium-dependent and sodium-independent conditions; phlorizin (Pz) 0.5 mM was used as specific inhibitor of SGLT1, phloretin (Pt) 0.5 mM as specific GLUT2 inhibitor and cytochalasin B (CB) 0.01 mM as GLUTs inhibitor. Since inhibitors were dissolved in dimethyl sulfoxide (DMSO) a final concentration of 0.1% DMSO was present in the uptake media where inhibitors were added. Caco-2/TC7 cells were pre-incubated with

pre-incubation medium (control) and pre-incubation media. After removing pre-incubation media, uptake was initiated by adding 500 µl of uptake media (control) or uptake media containing Pz, Pt, CB or DMSO. Uptake was terminated by washing cells three times with ice cold PBS. Cells were treated for radioactivity count as procedure described in section **4.3.2.3** Inhibitors of glucose transporters were tested throughout the *in vitro* Caco-2/TC7 cells assays.

Table 4.6 Reagents details for cell culture assays

Reagent	**Supplier details**
D-(+)-glucose	BDH Laboratory Supplies, cat. no. 101174Y
L-(-)-glucose	Santa Cruz Biotechnology, cat. no. sc-221793A
Glucose, D-[^{14}C(U)]	Perkin Elmer, cat. no. NEC042X050UC
Glucose, L-[1-^{14}C]	Perkin Elmer, cat. no. NEC478050UC
HEPES	Sigma Aldrich, cat. no. H3375
$CaCl_2$ 1 M	AMRESCO, LLC. cat. no. E506
BSA (bovine serum albumin)	Sigma Aldrich, cat. no. A9647
PBS (phosphate buffer solution)	Severn Biotech, 20-7400-10
SDS (sodium dodecyl sulphate)	Fisher, cat. no. 02674-25
Ecoscint A Scintillation Cocktail	National Diagnostics, LS-273
Phlorizin	Cayman Chemical Company, cat. no.11576
Phloretin	Santa Cruz Biotechnology, cat. no. sc-3548
Cytochalasin B	Santa Cruz Biotechnology, cat. no. sc-3519
DMSO (dimethyl sulfoxide)	Fisher Scientific CAS-No 67-68-5
Methanol	Fisher Scientific CAS-No 67-56-1
Ethanol	Fisher Scientific CAS-No 54-17-5

4.3.2.2.2 Time course assay

Before starting experiments Caco-2/TC7 cells were pre-incubated with complete media containing 3.2 mg/ml of blackcurrant polyphenols, times for pre-incubation were 0 (controls), 1 and 30 min and 3 hours. Following pre-incubation, complete media was removed and cells were washed once with room temperature KBS. Uptake was initiated by adding 500 µl of BE-free uptake media (control) or uptake media containing 3.2 mg/ml of blackcurrant polyphenols, after 10 min uptake was terminated by aspirating uptake media and washing the cells three times with 500 µl of ice cold PBS. Subsequently 500 µl of 0.2% SDS were added to each well and plates were incubated one hour at 37 °C for cellular lysis. Aliquots of 200 µl of cell lysate were added to 5 ml of scintillation liquid (National Diagnostic, Ecoscint A, LS-273) and radioactivity count was measured on a Liquid Scintillation Counter (Beckman Coulter, Inc. LS6500). Calculations for glucose uptake were made taking into account the specific activity of uptake media. Total glucose uptake was corrected for simple diffusion of L-glucose.

4.3.2.2.3 Dose response assays

Glucose uptake experiments were performed under sodium-dependent and sodium-independent conditions in order to identify acute effects of the fruit extracts on total glucose uptake (SGLT1- and GLUT-mediated) and GLUT-mediated glucose uptake. Before uptake started complete media was aspirated and cells were washed once with room temperature KBS. Cells were pre-incubated for 15 min with pre-incubation media; uptake was initiated by replacing pre-incubation media with uptake media. Uptake media contained, except for controls, increasing concentrations of blackcurrant or apple polyphenols. After 10 min the uptake media was aspirated and the transport process was stopped by washing three times each well with ice cold KBS, cells were treated for radioactivity count as previously described in this document.

4.3.2.2.4 Individual anthocyanins and anthocyanidins

Individual anthocyanins contained in the anthocyanin-rich blackcurrant extract and derived anthocyanidins (aglycones) were tested at estimated physiological (**Table 4.3**) concentrations and at 0.1 mM against 10 mM D-glucose and 1 mM D-glucose, respectively.

In the first set of experiments concentrations tested correspond with estimated physiological concentrations of individual compounds present in gut lumen after

consumption of the top dose drink used in GLU-BERRY study (600 mg ACN) (**Table 4.4**). Before uptake started complete media was aspirated and cells were washed once with room temperature KBS. Cells were pre-incubated for 15 min with pre-incubation media; uptake was initiated by replacing pre-incubation media with uptake media containing, except for control, different concentrations of individual anthocyanins, anthocyanidins, and solvents, combination of glycosides or combination of aglycones. After 10 min the uptake media was aspirated and the transport process was stopped by washing three times each well with ice cold KBS, cells were treated for radioactivity count as previously described in this document.

Further concentrations of individual compounds were tested in the Caco-2/TC7 system; in order to increase the anthocyanins/glucose ratio in uptake media different assay conditions were settled. Glucose concentration in uptake media was decreased to 1 mM and individual compounds in uptake media were tested at 0.1 mM given a ratio 1:10. Before uptake started complete media was aspirated and cells were washed once with room temperature KBS. Cells were pre-incubated for 15 min with pre-incubation media containing, except for control, 0.1 mM of individual anthocyanins, anthocyanidins or different concentrations of solvents. Uptake was initiated by replacing pre-incubation media with uptake media containing, except for control, 0.1 mM of individual anthocyanins, anthocyanidins or different concentrations of solvents. After 2 min the uptake media was aspirated and the transport process was stopped by washing three times each well with ice cold KBS, cells were treated for radioactivity count as previously described in this document.

4.3.3 *Xenopus laevis* oocytes injected to express SGLT1 system

4.3.3.1 In vitro synthesis of cRNA encoding rSGLT1

4.3.2.1.1 Linearising cDNA

Insertion of complementary DNA (cDNA) clones of rat SGLT1 (rSGLT1, ID: 7129017) into the pExpress-1 vector was previously done and verified in Dr Corpe's laboratory; full details were reported in the thesis of MSc Reshma Suresh. Analysis of the rSGLT1 plasmid generated showed the rSGLT1 clone inserted identical to published sequence and a plasmid mass of 6.5 kb, including 2.4 kb for the rSGLT1 inserted clone and 4.1 kb for the pExpress-1 vector (Suresh, 2013).

To obtain linearized DNA, rSGLT1-pExpress-1 cDNA was digested using the XhoI kit (New England BioLabs® Inc) following supplier's protocol. Mixtures were made up to a final volume of 50 µl with RNase-free water and incubated two hours in a bath water at 37 ^{o} C. Precipitation of linearized sample was done by adding 2.5 µl of EDTA (0.5 M), 5 µL of ammonium acetate (5 M) and 100 µl of ethanol (100%). Mixture was chilled at -20 ^{o} C for 30 min and centrifuged at 4 ^{o} C, 13000 rpm for 15 min. Supernatant was discarded, and 10 min were allowed for ethanol evaporation before pellet were re-suspended in 10 µL of RNase-free water. Linearized DNA concentration was measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, UK).

Following DNA digestion, linearization of rSGLT1-pExpress-1 cDNA was confirmed by agarose gel electrophoresis (**Figure 4.1**). Agarose gel 1% was made up of UltraPure Agarose (Life Technologies, UK), TBE buffer (**Table 4.7**) and stained with 5 µg Ethidium Bromide (Alfa Aesar, UK). 500 ng of cDNA and linearized DNA were complemented with 2 µl of Blue/Orange 6X Loading Dye (Promega, UK) and made up to 6 µl with RNase-free water. Samples were added to different wells in the electrophoresis gel and ran together with 20 µl of 1kb DNA Ladder (Promega) in a TBE buffer-filled box at 120 V for 90 min. Gel was visualised on a SynGene Genius Bioimaging system using GeneSnap, version 6.00.26 (Synaptics, UK). The electrophoresis showed bp size of uncut cDNA and linearised cDNA as expected (6.5 kb) although uncut cDNA ran lower than expected possible due to the presence of supercoiled DNA.

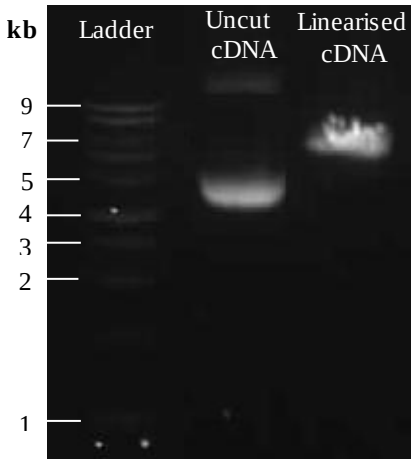


Figure 4.1 Electrophoresis of uncut cDNA and linearised cDNA of rSGLT1
After linearisation of cDNA with restriction enzymes XhoI samples alongside with 1 kb DNA ladder were analysed in agarose gel 1% stained with ethidium bromide. Electrophoresis showed bp size of uncut cDNA and linearised cDNA as expected (6.5 kb), although uncut cDNA ran lower than expected possible due to the presence of supercoiled DNA.

4.3.2.1.2 Synthesis of cRNA encoding rSGLT1 for oocyte microinjection

The transcription kit mMESSAGE mMACHINE® SP6 (Ambion^{TM}) was used to transcribe linearised DNA of rSGLT1 into complementary RNA (cRNA) encoding rSGLT1 according to manufacturer's protocol. For synthesis of cRNA 7.5 µg of linearised DNA were complemented with 3 µl of nuclease-free water, 10 µl of NTP/CAP solution, 2 µl of reaction buffer and 2 µl of SP6 enzyme mix. The mixture was gently mixed and incubated at 37 ^{o} C for 2 hr. For removing DNA template 1 µl of TURBO DNase was added to the sample, mixed and incubated at 37^{o}C for 15 min. Reaction was stopped by adding 30 µl of nuclease-free water and cRNA was precipitated by adding 30 µl of Lithium Chloride Precipitation Solution, mixtures were chilled at -20 ^{o}C for 45 min and centrifuged at 4 ^{o} C and 13000 rpm for 15 min in order to pellet the cRNA. After centrifugation the supernatant was carefully discarded by decantation and cRNA pellet was washed with 1 ml of 70% ethanol, mixture was centrifuged again at 4 ^{o} C, 13000 rpm for 15 min, supernatant was discarded and 10 min were allowed to ethanol evaporation before the cRNA pellet were re-suspended in 30 µl of nuclease-free water. Concentration of cRNA encoding rSGLT1 was measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, UK); sample was storage at -20 ^{o} C.

Size and integrity of the cRNA was verified by running a Nano chip electrophoresis with the Agilent 2100 Bioanalyzer using the RNA 6000 Nano kit (Agilent Technologies, Germany). Samples were analysed by Erick Nasser in the Genomic Centre of King's College London, before analysis sample was diluted to a final concentration of ~100 ng/µl. Results in the gel image (**Fig 4.2 A**) and electropherogram (**Fig 4.2 B**) from the cRNA Nano chip electrophoresis indicate a structure in the 2.4 kb range as expected for the rSGLT1 inserted clone. Although results suggest a possible secondary structure by the presence of two bands and two peaks instead of a single one, validation assays of the system showed the clone works as expected in oocytes injected cRNA encoding rSGLT1 (Suresh, 2013, Munoz Sandoval, 2015).

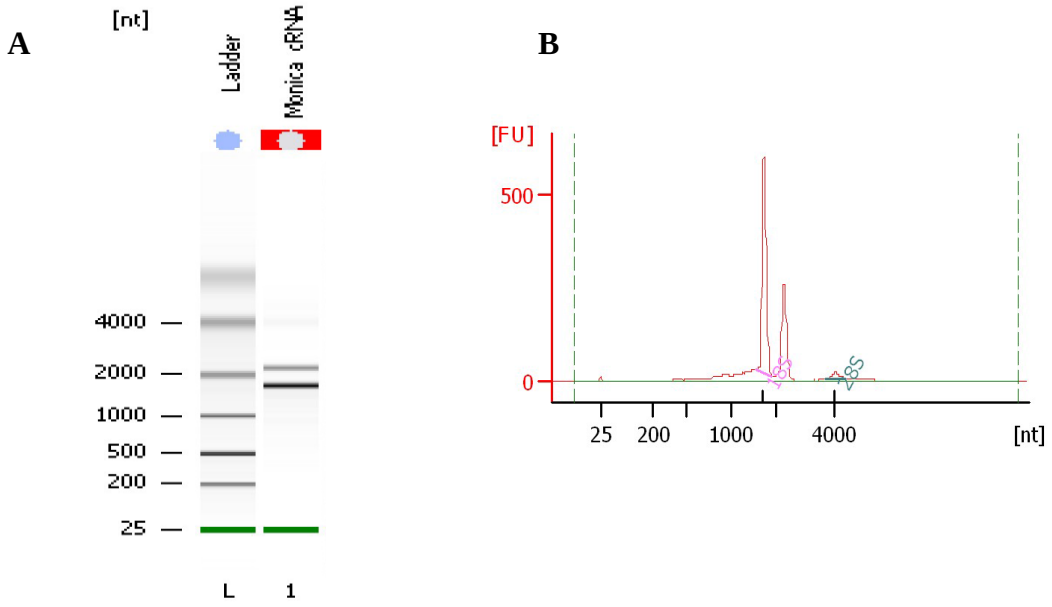


Figure 4.2 Nano chip electrophoresis of rSGLT1 cRNA
Transcription of linearised cDNA of rSGLT1 into cRNA was analysed for purity and integrity. **A**) Gel image of rSGLT1 cRNA. L, ladder; 1, cRNA of rSGLT1. The presence of two bands indicate an incomplete transcription reaction, however the band above 2000 [nt] pinpoint the presence of a structure within the expected range (2.4 kb for inserted rSGLT1 clone). **B**) Electropherogram of rSGLT1 cRNA. The presence of two peaks suggests the presence of a secondary structure however the peak in the 2000 [nt] range indicate a structure within the predicted size.

4.3.3.2 Expression of SGLT1 transporter in oocytes

4.3.3.2.1 Oocyte isolation

Batches of *Xenopus laevis* oocytes were provided by the European *Xenopus* Resource Centre of the University of Portsmouth. After arrival ovary sacs containing oocytes were placed in petri dishes and washed two to three times with calcium-free MBS media (**Table 4.8**) before start the manual separation of the ovary lobes and releasing of individual using watchmaker forceps. Released individual oocytes were washed three times with MBS Ca^{2+} free-media and incubated in 0.2% Collagenase IV from *Clostridium Histolitycum* (Sigma Aldrich, UK) contained in calcium-free MBS media; petri dishes were wrapped in aluminium foil, in order to protect oocytes from light, and placed on a shaker at room temperature and 4 rpm for 30 min. After incubation manual dissection with forceps was carefully done, subsequently media was removed and incubation with collagenase followed by oocyte separation was repeated. After collagenase incubation and manual dissection oocytes were washed two times with

MBS media (with Ca^{2+}), in order to stop the collagenase action, and incubated in fresh MBS media.

Selection of healthy oocytes was done using a Pasteur pipette, perfectly spherical oocytes with ~1 mm of diameter without any follicular attachment and with a clear division of the animal (black) and vegetal (white) pole (**Figure 4.3**) were selected using a Stereo Microscope (Leica StereoZoom GZ6) equipped with a cold light source (Leica CLS 100X). After selection oocytes were transferred into 6-well culture plates (NUNC, UK) and incubated in MBS media supplemented with 1 mM sodium pyruvate (GE Healthcare, UK). Plates were wrapped in aluminium foil and incubated at 19 ^{o} C (LABCOLD Incubator, UK) until microinjection.

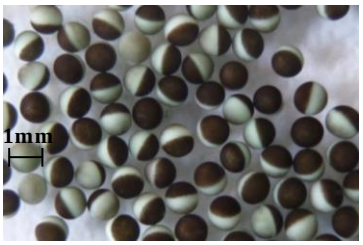


Figure 4.3 Healthy oocytes

Table 4.7 Solutions used in the oocyte system

Solution	Composition
TBE buffer (10X)	0.90 M Tris, 0.89 M Boric acid and 20 M EDTA
MBS (modified Barth's saline media)	88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 10 mM HEPES, 0.41 mM $CaCl_2$, 100U/100 µg penicillin/streptomycin, 0.05 mg/L tetracycline and 0.1 mg/ml gentamicin. pH 7.5

4.3.3.2.2 Oocyte microinjection

Before injecting, oocytes were inspected for irregular shaped or damaged oocytes to be discarded. Healthy oocytes were collocated in the oocyte chamber, consisting of a petri dish filled with MBS media and a polypropylene mesh (0.8 mm pores) attached to the bottom, the mesh allows oocyte to be immobilised during microinjections.

Microinjections of cRNA were done with a Nanoject II injector (Drummond Scientific Company) with a glass needle attached (**Figure 4.4**). With help of a Stereo Microscope equipped with cold light, 36.8 nL of rSGLT1 cRNA (1.2-1.5 µg/ml) were injected to each oocyte. For microinjections glass needles were pulled from capillary glass pipettes (Drummond Scientific Company, US) using a P87 Flaming/Brown Micropipette Puller (Sutter Instrument Company, US) with the following parameters; heat 749, pull 63, velocity 45 and time 210. Prior to injection needles were opened by carefully breaking off the tip with forceps, tip needle was 10-15 mm in length. Needles were loaded with 3.5 µL of cRNA at the bottom of the tip followed by 12 µL of mineral oil in the barrel, 0.5-20 µL Microloader pipette tips (Eppendorf, US) were used to fill the needles. The needle was then attached to the Nanoject II injector and the injector was placed at 40 ^{o} angle with respect to the oocyte chamber. Injected oocytes and healthy non-injected oocytes were transferred to 6-well plates filled with MBS media supplemented with 1 mM sodium pyruvate, plates were wrapped in aluminium foil and incubated at 19 ^{o} C until assay day. Oocytes were checked daily and removed into fresh media when necessary, transport assays were carried out at fourth day post injection, allowing time for the cRNA translation and expression of mature protein (SGLT1 transporter) into the oocyte plasma membrane. Non-injected oocytes were used as controls in the uptake assays, and were named as shams in this document.

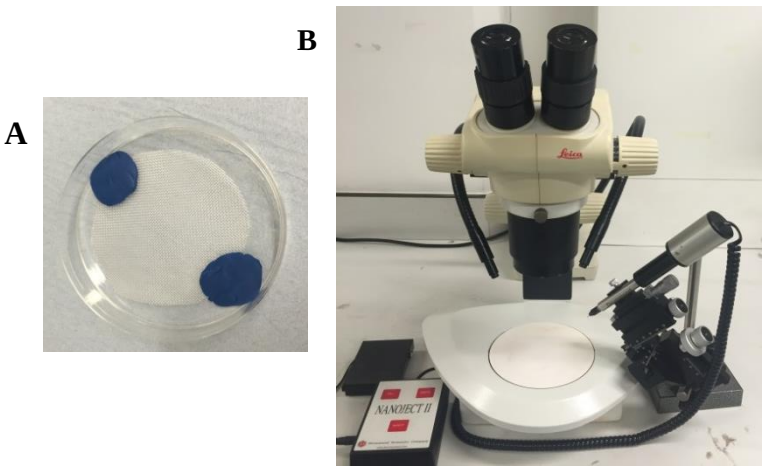


Figure 4.4 *Xenopus* oocyte microinjection equipment
A) Oocyte injection chamber with polypropylene mesh (0.8 mm pores) attached for holding oocytes. **B)** Microinjection workstation with stereo microscope, cold light source power and microinjector.

4.3.3.3 Glucose transport assays

Conditions for glucose uptake assays were previously established in the laboratory by MSc Reshma Suresh and MRes Diana Munoz Sandoval. Ten oocytes injected to express SGLT1 and ten sham oocytes were used for every condition tested. For glucose transport assays uptake media contained 10 mM D-glucose and 0.5 µCi/ml Glucose D-[^{14}C(U)] as tracer (**Table 4.1**). MBS was used as uptake media and PBS as wash media; uptake period in all experiments was 10 minutes. Throughout assays uptake was started by aspirating incubation media and adding 500 µl of uptake medium and terminated by aspirating uptake media and washing three times with 4 ml of ice cold PBS. Subsequently each oocyte was solubilised in 500 µl of 10% SDS contained in a scintillation vial prior to the addition of 4 ml of Ecoscint A, vials were vigorously shaken by hand before radioactivity count. Radioactivity count was measured on a Liquid Scintillation Counter (Beckman Coulter, Inc. LS6500). Calculations for glucose uptake were made taking into account the specific activity of uptake media and total glucose uptake was corrected for D-Glucose diffusion in sham oocytes.

4.3.3.3.1 Inhibition assays in oocytes expressing SGLT1

To ensure the oocyte system was functioning as expected phlorizin (Pz), a well-known competitive SGLT1 inhibitor and phloretin (Pt), a non-competitive SGLT1 inhibitor were tested on the oocytes injected to express rSGLT1 and sham oocytes. Pz 0.5 mM and Pt 0.5 mM were dissolved in dimethyl sulfoxide (DMSO) therefore a concentration of 0.1% DMSO was also tested in oocytes. After remove incubation media, uptake was initiated by adding 500 µl of uptake medium (control) or uptake media complemented with DMSO, Pz or Pt. After 10 min uptake was terminated and oocytes were washed and treated as procedure described in section 4.3.2.3. Pz 0.5 mM and 0.1% DMSO were tested throughout the *in vitro* oocytes assays.

4.3.3.3.2 Dose response assays with anthocyanin-rich blackcurrant extract and polyphenol-rich apple extract (AE2) in oocytes expressing SGLT1

Before uptake started incubation medium was aspirated, uptake was initiated by adding uptake media. Uptake media contained, except for control, increasing concentrations of blackcurrant (BE) or apple extract (AE2), after 10 min the uptake media was aspirated and the transport process was stopped by washing oocytes three times with ice cold

PBS. Oocytes were treated for radioactivity count as previously described in section 4.3.2.3. Concentrations of BE and AE2 tested in the oocyte system were the physiological range estimated to be present in the small intestine after ingestion of test drinks on human studies and additional higher and lower concentrations.

4.3.4 Statistical analysis

All results were expressed as mean ± SEM. Statistical analysis was done using the software GraphPad Prism 6 for Windows (GraphPad software, CA, USA). All data was tested for normality with D-Agostino & Pearson omnibus test. Experiments with normally distributed data were analysed by one-way analysis of variance with Dunnett's multiple comparison post hoc test or by unpaired t-test. Experiments with non-normally distributed data were analysed by Kriskal-Wallis test with multiple comparison Dunn's post hoc adjustment or by Mann-Whitney test. IC_{50} were estimated by non-linear regression analysis using the software SigmaPlot 13 for windows (Systat Software Inc. CA, USA).

4.4 Results

Results showed in this section represent pooled data of three or more assay repetitions testing anthocyanin-rich blackcurrant extract and polyphenol-rich apple extracts on Caco-2 cells and oocytes systems. Validation details of the Caco-2/TC7 cells and *Xenopus laevis* oocytes systems implemented by the research group were reported in the theses of Dr Patrick O'Brien (O'Brien, 2015) and MRes Diana C Munoz Sandoval (Munoz Sandoval, 2015), respectively.

4.4.1 Effects of glucose transporter inhibitors on Caco-2/TC7 system

All through the period of experimentation on the Caco-2 cells system specific inhibitors of glucose transporters; phlorizin, phloretin and cytochalasin B which inhibit SGLT1, GLUT2 and GLUT 1-4, 7, 8 respectively, showed the *in vitro* system functioning as expected. Total glucose uptake was decreased by specific glucose transporter inhibitors (P<0.0001): phlorizin decreased total glucose uptake by 23%, phloretin by 22% and cytochalasin B by 42% (**Figure 4.5 A**). Specific glucose transporter inhibitors were also tested under sodium independent conditions. As expected GLUT-mediated glucose uptake was decreased by GLUTs inhibitors, phloretin and cytochalasin B (P<0.0001), but not by phlorizin. Phloretin decreased GLUT-mediated glucose uptake by 14% and cytochalasin B by 40% (**Figure 4.5 B**).

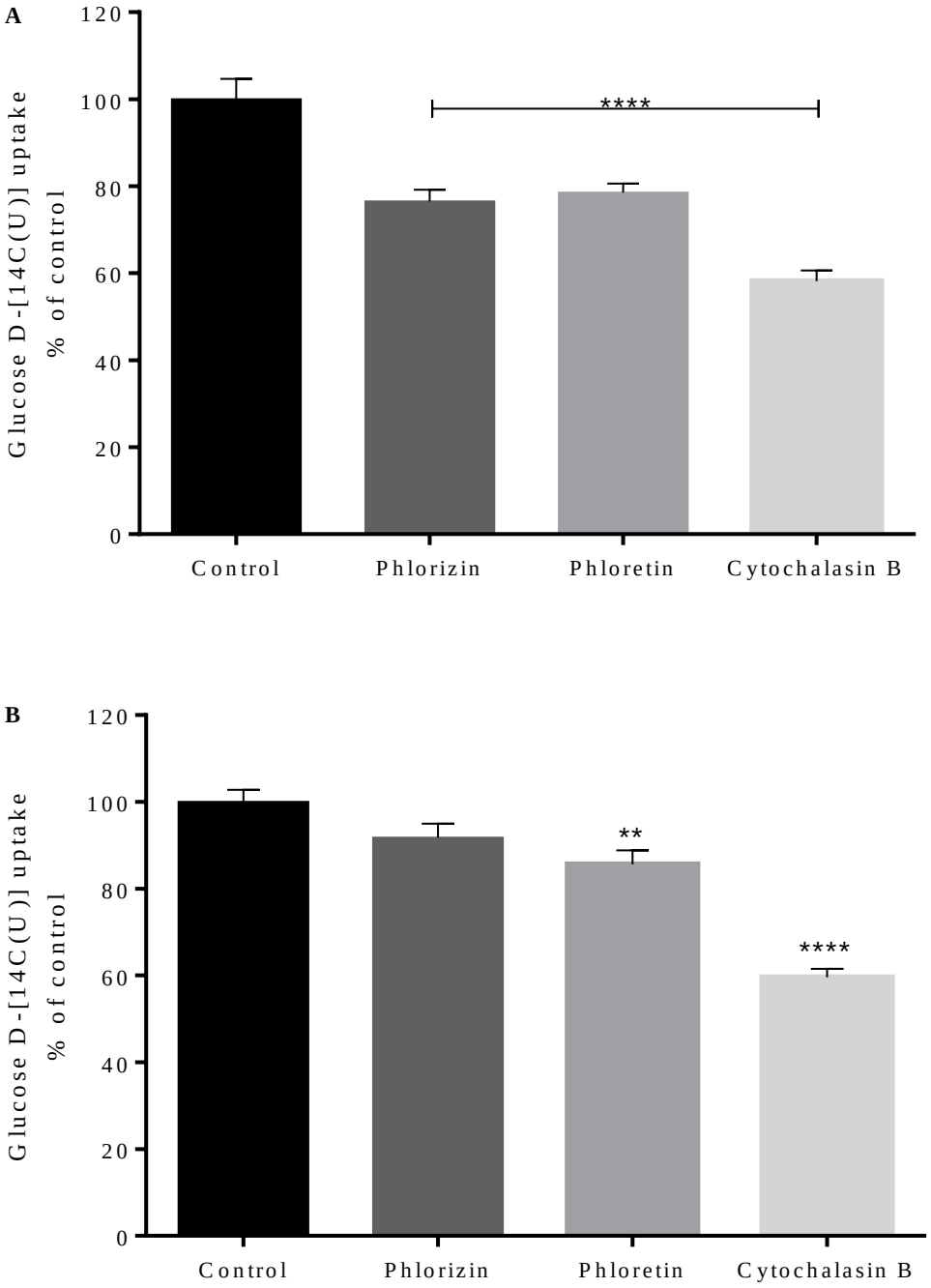


Figure 4.5 Inhibition of total and GLUT-mediated glucose uptake by phlorizin, phloretin and cytochalasin B

Caco-2/TC7 cells were treated with phlorizin 0.5 mM, phloretin 0.5 mM and cytochalasin B 0.01 mM contained in uptake media, control was supplemented with dimethyl sulfoxide 0.1 %. Values were corrected for simple diffusion by subtracting L-glucose uptake. Data are presented as mean ± SEM (n=4-5). One-way analysis of variance followed by multiple comparisons against control with Dunnett's adjustment. **A)** Total glucose uptake: ****P<0.0001 for the difference between control and phlorizin, phloretin and cytochalasin B. **B)** GLUT-mediated glucose uptake: **P<0.005 for the difference between control and phloretin; ****P<0.0001 for the difference between control and cytochalasin B.

4.4.2 Effects of anthocyanin-rich blackcurrant extract (BE) on glucose uptake

4.4.2.1 Time course assay with BE

Time course assay showed a statistically significant inhibition of total glucose uptake on Caco-2/TC7 cells pre-incubated with 3.2 mg of blackcurrant polyphenols (P<0.0001) (**Figure 4.6**). Concentration of blackcurrant polyphenols present in pre-incubation and uptake media is fourfold the dose estimated to be present in gut lumen when top dose drink (600 mg ACN) was consumed by participants in human study (Chapter 3). There was an acute effect of blackcurrant polyphenols on total glucose uptake, uptake was significantly decreased after 0, 1, 30 min and 3 h pre-incubation (P<0.0001). Significant inhibitory effect following 0 and 1 min pre-incubation treatments served for establishing assay conditions for testing acute effects of rich-polyphenols fruit extracts on glucose uptake. All the following assays were performed with no fruit extract added in pre-incubation media.

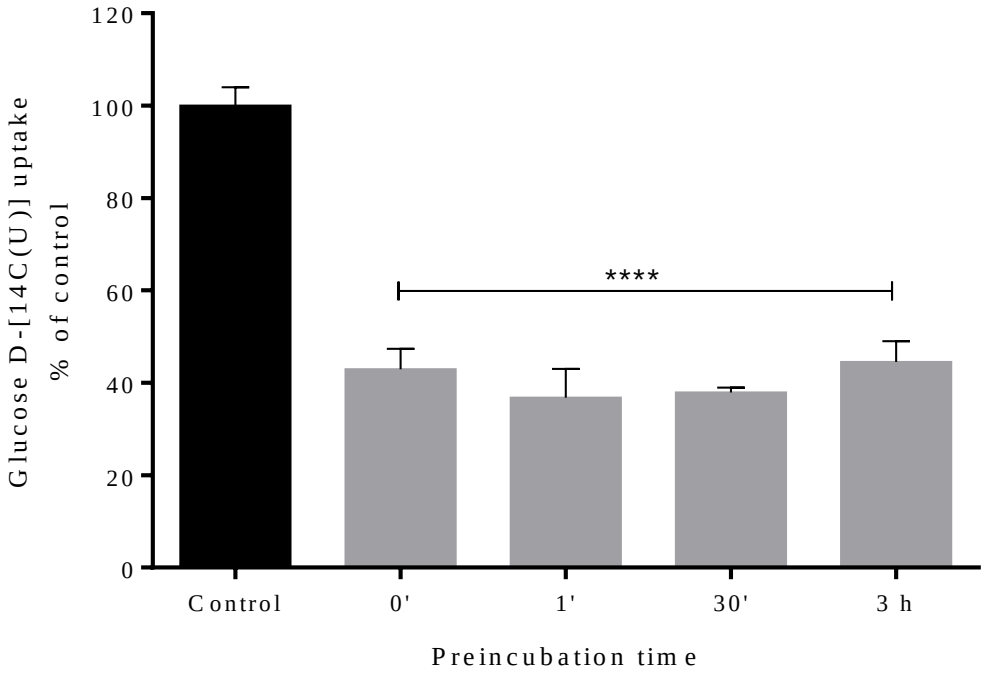


Figure 4.6 Total glucose uptake inhibition after treatment with blackcurrant extract
Caco-2/TC7 cells were pre-incubated with 3.2 mg/ml of blackcurrant polyphenols before glucose uptake, uptake media contained, except for control, 3.2 mg/ml of blackcurrant polyphenols. Values were corrected for simple diffusion by subtracting L-glucose uptake. Data are presented as mean ± SEM (n=3). One-way analysis of variance followed by multiple comparisons against Control with Dunnett's adjustment: ****P<0.0001 for the difference between Control and 0', 1', 30' and 3 h pre-incubation.

4.4.2.2 Acute effects of BE on glucose uptake

There was an acute effect of blackcurrant polyphenols on total glucose uptake, following 10 min uptake in the presence of different concentrations of blackcurrant polyphenols total glucose uptake was inhibited in a dose-responsive manner ($P<0.0001$) (**Figure 4.7A**). There was a significant reduction on total glucose uptake after treatment 0.2 mg/ml ($P<0.005$), and a highly significant reduction following treatments 0.4, 0.8, 1.6 and 3.2 mg/ml ($P<0.0001$). The dose-response assay was performed under sodium-independent condition in order to assess inhibitory effects by blackcurrant polyphenols on GLUT-mediated glucose uptake. There was a dose-response inhibition on GLUT-mediated glucose uptake, following 10 min uptake in the presence of different concentrations of blackcurrant polyphenols ($P<0.0001$) (**Figure 4.7B)**. There was a significant reduction on GLUT-mediated glucose uptake following treatments 0.2, 0.4, 0.8 and 1.6 mg/ml ($P<0.0001$). The estimated physiological concentration corresponding to the effective dose in the human study (GLU-BERRY study) was 0.8 mg/ml and inhibited 66 and 60 % of the total and GLUT-mediated glucose uptake, respectively.

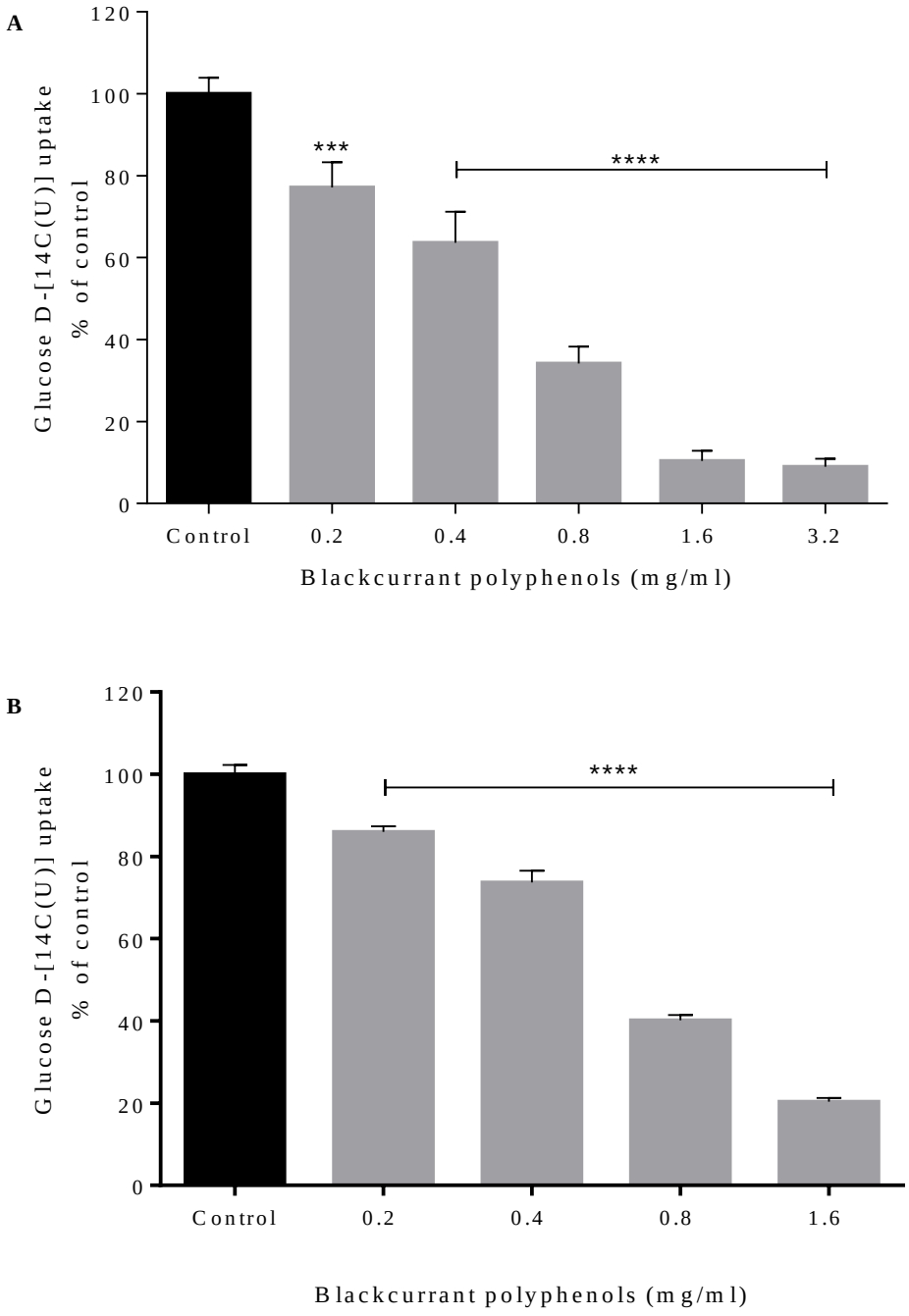


Figure 4.7 Acute effects of increasing concentrations of blackcurrant polyphenols on total and GLUT-mediated glucose uptake

Caco-2/TC7 cells were treated with increasing concentrations of blackcurrant polyphenols contained in uptake media, except for control (0 mg/ml). Values were corrected for simple diffusion by subtracting L-glucose uptake. Data are presented as mean ± SEM (n=3-5). One-way analysis of variance followed by multiple comparisons against control (0 mg/ml) with Dunnett's adjustment: **A)** Total glucose uptake: ****P*<0.001 for the difference between Control and 0.2 mg/ml; *****P*<0.0001 for the difference between Control and 0.4, 0.8, 1.6 and 3.2 mg/ml. **B)** GLUT-mediated glucose uptake: *****P*<0.0001 for the difference between Control and 0.2, 0.4, treatments.

Concentrations of blackcurrants phenolic necessary to inhibit by half (IC_{50}) total and GLUT-mediated glucose uptake were estimated. Blackcurrant polyphenols under sodium-dependent conditions showed and IC_{50}= 0.51 ± 0.09 mg/ml (mean ± SE) (**Figure 4.8A**) and under sodium-independent conditions IC_{50}= 0.63 ± 0.07 mg/ml (**Figure 4.8B**). Findings suggest a slightly greater inhibition on total glucose uptake than GLUT-mediated glucose uptake by blackcurrant polyphenols.

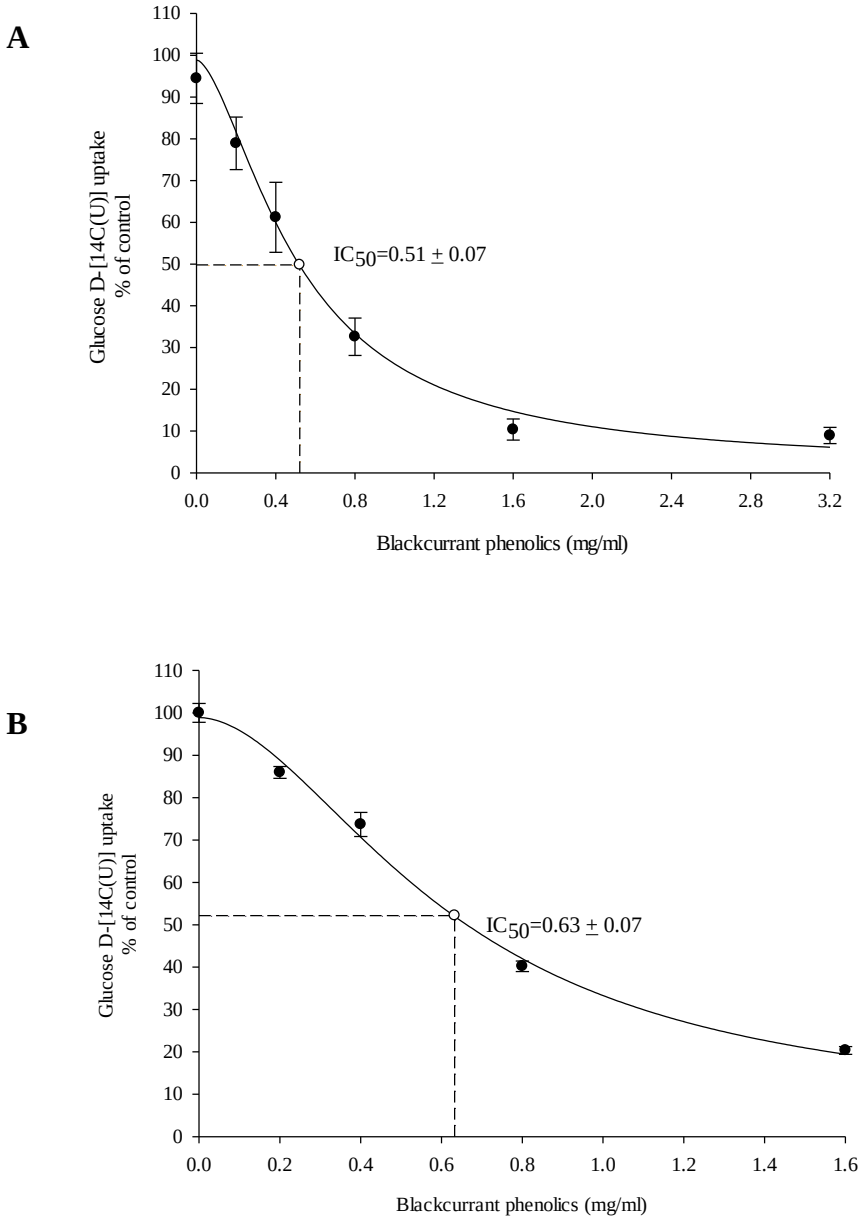


Figure 4.8 Blackcurrant polyphenols IC_{50} for total and GLUT-mediated glucose uptake Effect of blackcurrant polyphenols on total (sodium-dependent) and GLUT-mediated glucose uptake (sodium-independent). Nonlinear regression analysis showed different values for IC_{50}s under sodium-dependent **(A)** and sodium-independent conditions **(B)**.

4.4.2.3 Acute effect of individual anthocyanins and anthocyanidins contained in BE

Individual anthocyanins and anthocyanidins present in the anthocyanin-rich blackcurrant extract and combinations of them did not decrease total glucose uptake (10 mM D-glucose, 10 min). There was no inhibitory effect when testing physiological concentrations estimated to be present in gut lumen after consumption of top dose drink in GLU-BERRY study (600 mg ACN) (**Figure 4.9**). Since individual compounds were dissolved in different solvents and therefore their control was not the same data were normalised and expressed as percentage relative to control.

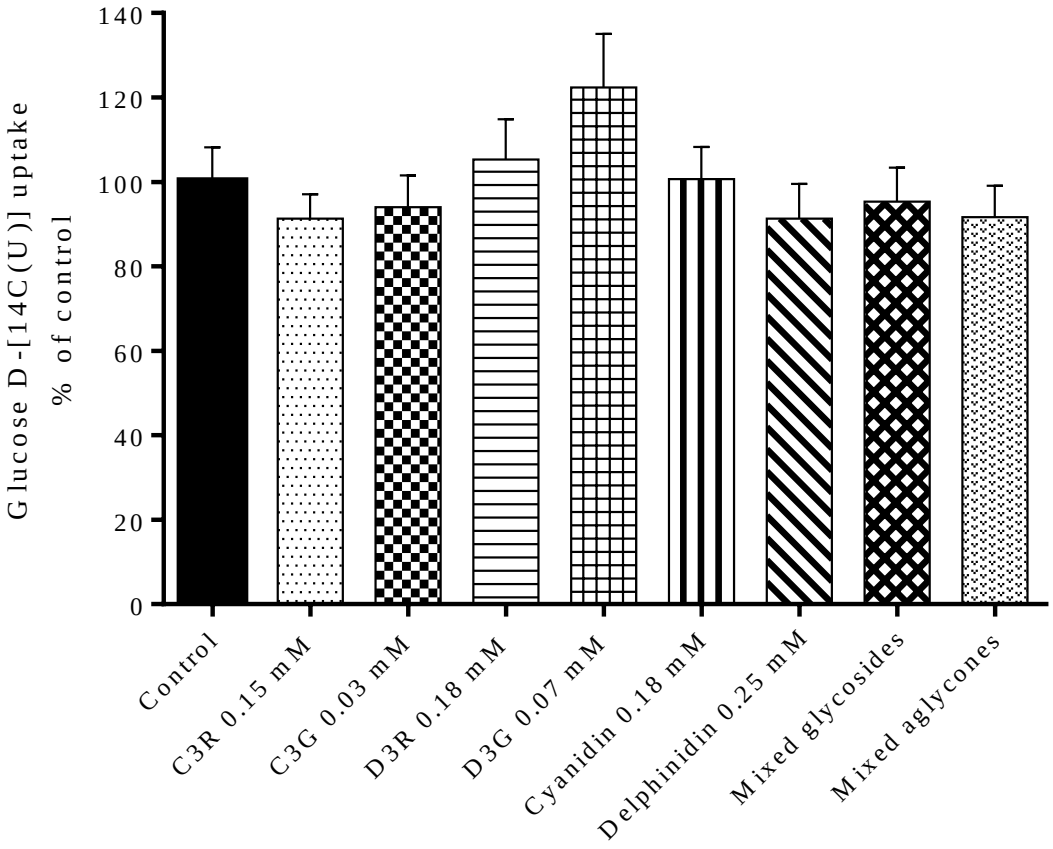


Figure 4.9 Acute effects of estimated physiological concentrations of individual anthocyanins and anthocyanidin on total glucose uptake

Caco-2/TC7 cells were treated with estimated physiological concentrations of anthocyanins, anthocyanidins and combinations contained in uptake media (10 mM D-glucose); except for Control (uptake media supplemented with solvent). C3R, cyanidin-3-rutinoside; C3G, cyanidin-3-glucoside; D3R, delphinidin-3-rutinoside; D3G, delphinidin-3-glucoside. Values were corrected for simple diffusion by subtracting L-glucose uptake. Data are presented as normalised means expressed as percentage relative to control ± SEM (n=3).

To test whether increased concentrations of inhibitors related to substrate further modifications in glucose uptake medium concentration (1 mM D-glucose, 2 min); anthocyanins/anthocyanidins concentrations (0.1 mM) were implemented. However increased ratio inhibitor/substrate and adding anthocyanins in pre-incubation media in addition to uptake media did not affect the rate of glucose uptake following treatment with individual anthocyanins and anthocyanidins (**Figure 4.10**). There was no decrease in total glucose uptake after treatments. Data are presented as normalised means expressed as percentage relative to control.

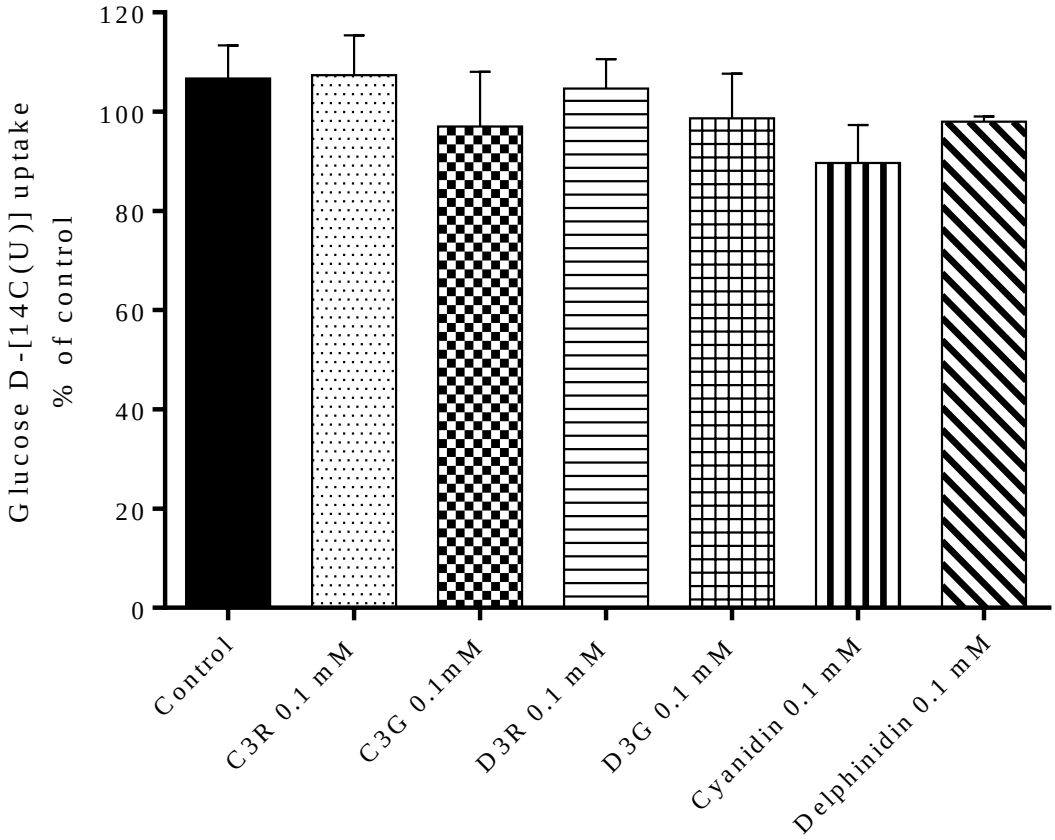


Figure 4.10 Acute effects of individual anthocyanins and anthocyanidin 0.1 mM on total glucose uptake

Caco-2/TC7 cells were treated with individual anthocyanins and anthocyanidins (0.1 mM) contained in pre-incubation and uptake media (1 mM D-glucose), except for Control (uptake media was supplemented with solvent). C3R, cyanidin-3-rutinoside; C3G, cyanidin-3-glucoside; D3R, delphinidin-3-rutinoside; D3G, delphinidin-3-glucoside. Values were corrected for simple diffusion by subtracting L-glucose uptake. Data are presented as normalised mean expressed as percentage relative to control ± SEM (n=3). There were no differences between treatments.

4.4.3 Effects of polyphenol-rich apple extract (AE1) on glucose uptake

There was an acute effect of apple polyphenols (using the extract from the GLU-APP study) on glucose uptake, dose response assays in the presence of different concentrations of apple polyphenols showed an inhibition in total glucose uptake ($P<0.0001$) (**Figure 4.11A**). There was a statistically significant inhibition in total glucose uptake after treatment 0.15 mg/ml ($P<0.05$), 0.3 mg/ml ($P<0.005$), and a highly significant reduction following treatments 0.5 mg/ml ($P<0.001$) and 1.0 mg/ml ($P<0.0001$). Dose-response assay was performed under sodium-independent condition in order to assess inhibitory effects by apple polyphenols (AE1) on GLUT-mediated glucose uptake. There was an inhibition on GLUT-mediated glucose uptake following treatment with apple polyphenols ($P<0.0001$) (**Figure 4.11B**). There was no inhibitory effect after 0.07 and 0.15 mg/ml conditions, there was a significant decrease on GLUT-mediated glucose uptake following treatments 0.3 mg/ml ($P<0.05$) and 0.5 and 1.0 mg/ml ($P<0.001$). The estimated physiological concentration corresponding to the top dose in the human study (GLU-APP study) was 0.3 mg/ml and inhibited 30 and 18% of the total and GLUT-mediated glucose uptake, respectively.

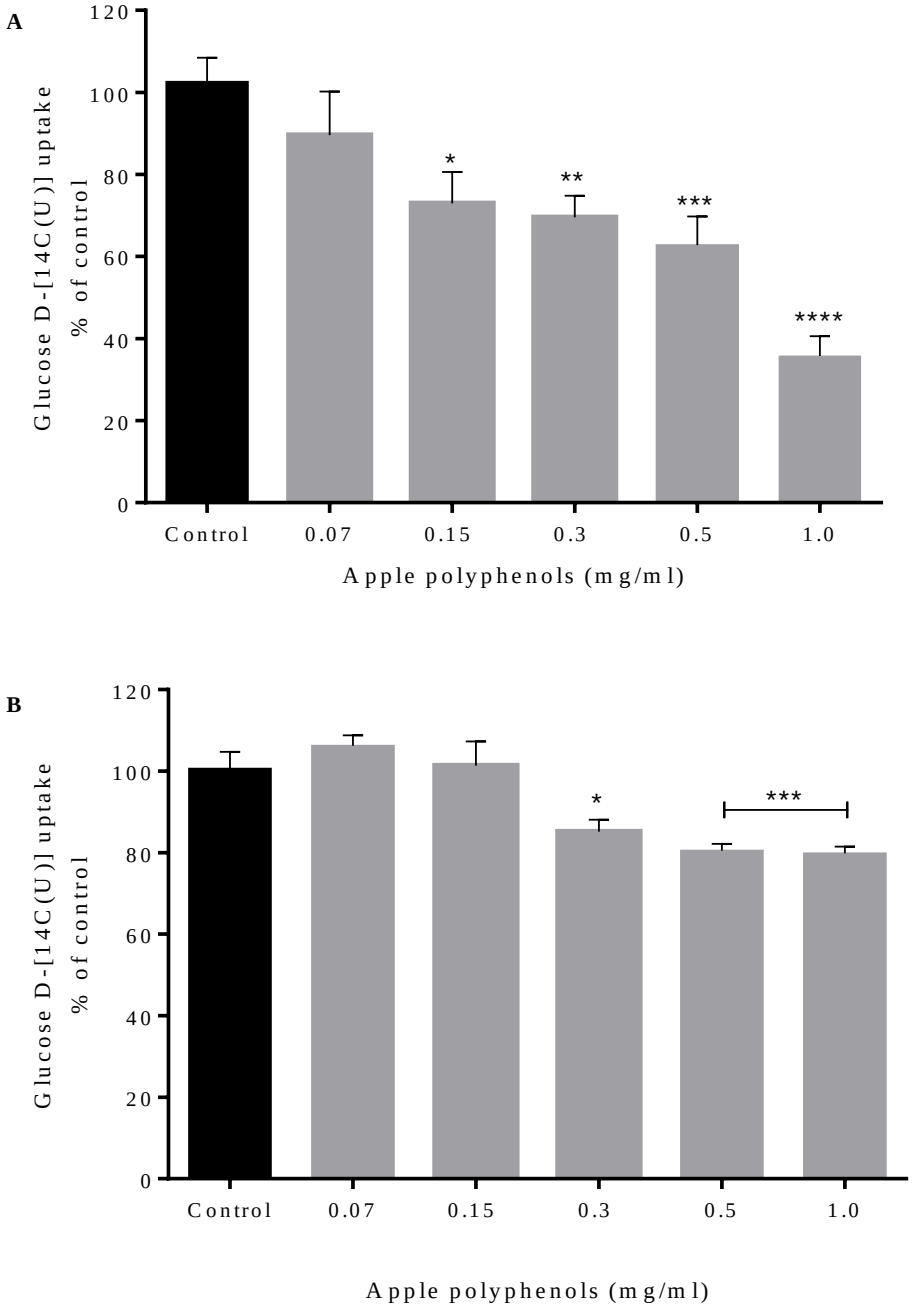


Figure 4.11 Acute effects of increasing concentrations of apple polyphenols (AE1) on total and GLUT-mediated glucose uptake

Caco-2/TC7 cells were treated with increasing concentrations of apple polyphenols contained in uptake media, except for Control (0 mg/ml). Values were corrected for simple diffusion by subtracting L-glucose uptake. Data are presented as mean ± SEM (n=3). One-way analysis of variance followed by multiple comparisons against Control with Dunnett's adjustment: **A)** Total glucose uptake: *P<0.05 for the difference between Control and 0.15 mg/ml, ***P*<0.005 for the difference between Control and 0.3 mg/ml; ****P*<0.001 for the difference between Control and 0.5 mg/ml; *****P*<0.0001 for the difference between Control and 1.0 mg/ml. **B)** GLUT-mediated glucose uptake: *P<0.05 for the difference between Control and 0.3 mg/ml; ****P*<0.0005 for the difference between Control and 0.5, and 1 mg/ml treatments.

4.4.4 Effects of polyphenol-rich apple extract (AE2) on glucose uptake

There was an acute effect of polyphenols in this second apple extract (used in the GLU-FRU study) on glucose uptake, dose response assays in the presence of different concentrations of apple polyphenols showed an inhibition on total glucose uptake (P<0.0001) (**Figure 4.12A**). There was a highly significant inhibition on total glucose uptake following treatments 0.15, 0.3, 0.5, 1.0 and 2.0 mg/ml (P<0.0001). Dose-response assays were performed under sodium-independent condition in order to assess inhibitory effects by apple polyphenols on GLUT-mediated glucose uptake. There was an inhibition on GLUT-mediated glucose uptake following treatment with apple polyphenols (AE2) (P<0.0001) (**Figure 4.12B**). There was no inhibitory effect after 0.07 mg/ml treatment but there was a highly significant decrease on GLUT-mediated glucose uptake following treatments 0.15, 0.3 and 0.5 mg/ml (P<0.0001). The estimated physiological concentrations corresponding to effective doses in the human study (GLU-FRU study) were 0.3 and 0.5 mg/ml and inhibited 51 and 67% and 46 and 62% of the total and GLUT-mediated glucose uptake, respectively.

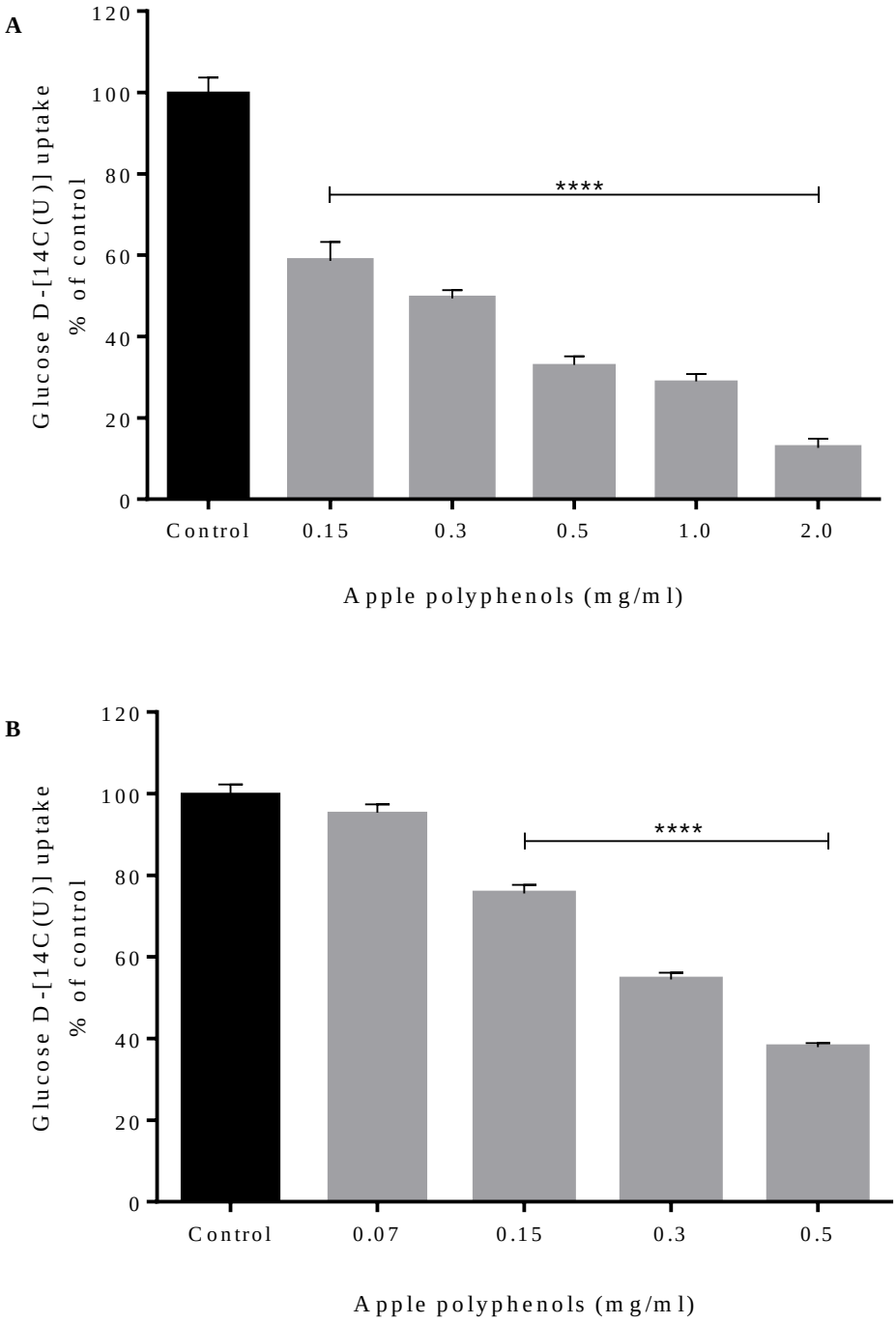


Figure 4.12 Acute effects of increasing concentrations of apple polyphenols (AE2) on total and GLUT-mediated glucose uptake

Caco-2/TC7 cells were treated with increasing concentrations of apple polyphenols contained in uptake media, except for Control (0 mg/ml). Values were corrected for simple diffusion by subtracting L-glucose uptake. Data are presented as mean ± SEM (n=3-5). One-way analysis of variance followed by multiple comparisons against Control with Dunnett's adjustment: **A)** ****P<0.0001 for the difference between Control and 0.15, 0.3, 0.5, 1.0 and 2.0 mg/ml treatments. **B)** GLUT-mediated glucose uptake: ***P<0.0005 for the difference between Control and 0.15, 0.3 and 0.5 mg/ml treatments.

4.4.5 Oocytes injected to express rSGLT1 system

All through the period of experimentation on the oocyte system phlorizin (dissolved in DMSO), a well-known SGLT1 inhibitor showed the *in vitro* system functioning as expected. Glucose uptake was decreased 85% and 59% by phlorizin and phloretin (0.5 mM), respectively when compared to 0.1% DMSO treatment (P<0.0001), as expected 0.1% DMSO (phlorizin and phloretin solvent) did not decreased glucose uptake (**Figure 4.13**).

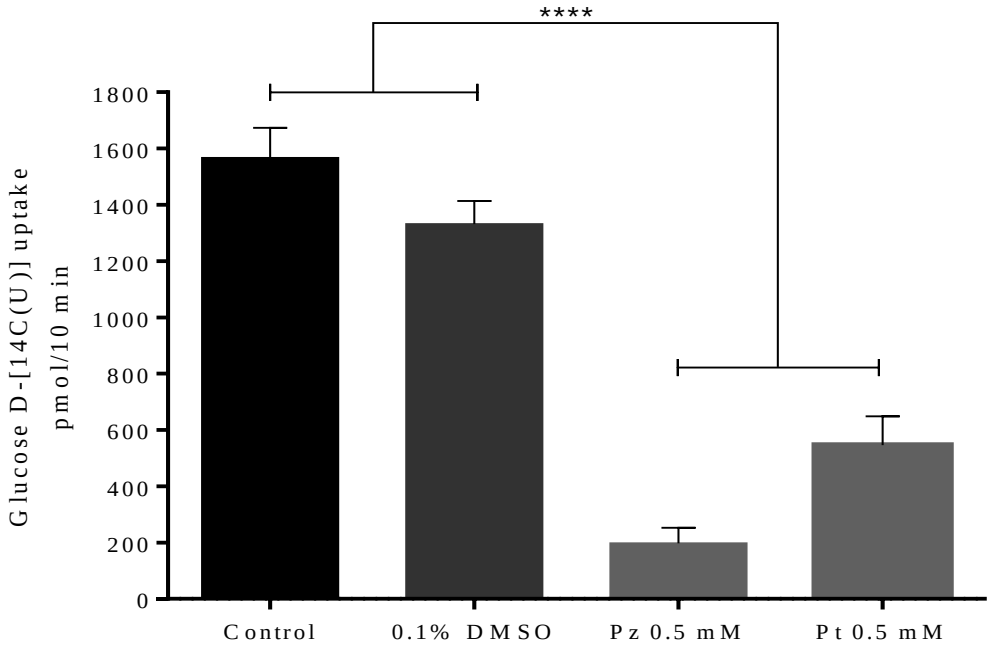


Figure 4.13 Inhibition of glucose uptake on oocytes expressing rSGLT1 by phlorizin and phloretin

Oocytes injected to express rSGLT1 were treated with DMSO, Pz or Pt, contained in uptake media, except for control. DMSO, dimethyl sulfoxide; Pz, phlorizin; Pt, phloretin. Data are presented as mean ± SEM (n=3). Values were corrected by subtracting sham oocytes uptake. One-way analysis of variance followed by multiple comparisons against controls with Dunnett's adjustment ****P<0.0001 for the difference between 0.1% DMSO, Pz 0.5mM and Pt 0.5 mM.

4.4.6 Effects of blackcurrant polyphenols (BE) on oocytes injected to express rSGLT1

Dose-response assays on oocytes expressing rSGLT1 showed an inhibition on glucose uptake following treatment with blackcurrant polyphenols (P<0.0001). There was a highly significant decreased on glucose uptake after treatments 0.1, 0.2, 0.4, 0.8 and 1.6 mg/ml (P<0.0001) (**Figure 4.14**).

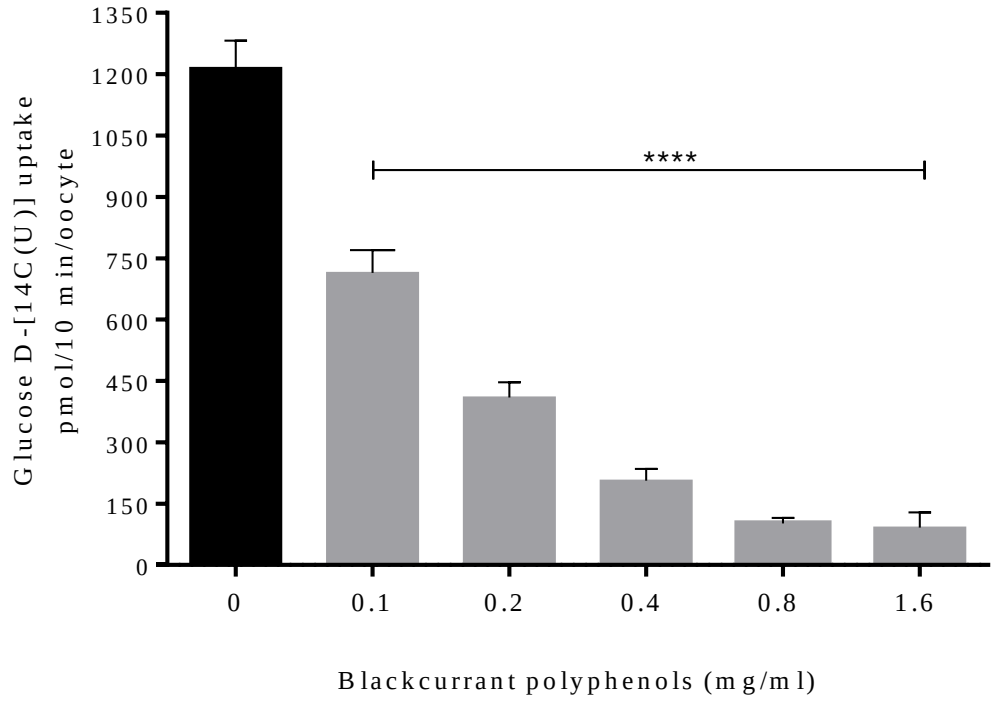


Figure 4.14 Effects of blackcurrant polyphenols on glucose uptake on oocytes expressing rSGLT1

Oocytes injected to express rSGLT1 were treated with increasing concentrations of blackcurrant polyphenols. Values were corrected by subtracting sham oocytes uptake. Data are presented as mean ± SEM (n=3). One-way analysis of variance followed by multiple comparisons against control with Dunnett's adjustment ****P<0.0001 for the difference between 0 mg/ml and 0.1, 0.2, 0.4, 0.8 and 1.6 mg/ml.

Concentration of blackcurrants polyphenols necessary to inhibit by half (IC_{50}) glucose uptake was 0.12 ± 0.003 mg/ml (mean ± SE) (**Figure 4.15**).

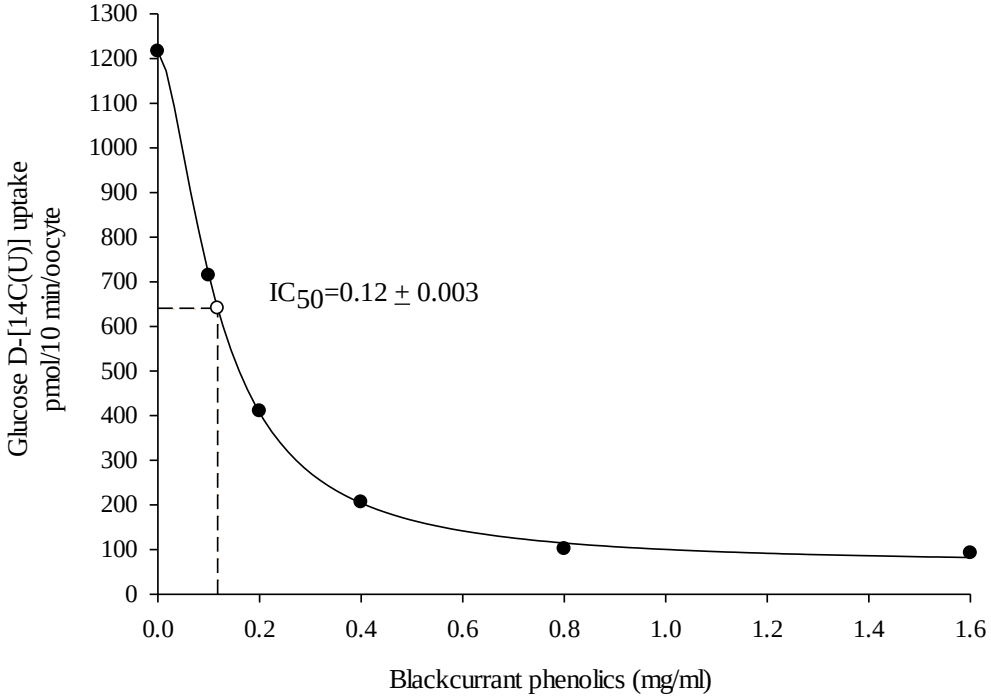


Figure 4.15 Blackcurrant polyphenols IC_{50} for glucose uptake on oocytes expressing rSGLT1

Effects of blackcurrant polyphenols on glucose uptake after dose response assays were used to calculate IC_{50} value. Nonlinear regression analysis showed a low value for IC_{50}.

4.4.7 Effects of apple polyphenols (AE2) on oocytes injected to express SGLT1

Dose-response assays on oocytes expressing rSGLT1 showed an inhibition on glucose uptake following treatment with apple polyphenols (P<0.0001). There was a highly significant decrease in glucose uptake after treatments 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 mg/ml (P<0.0001) (**Figure 4.16**).

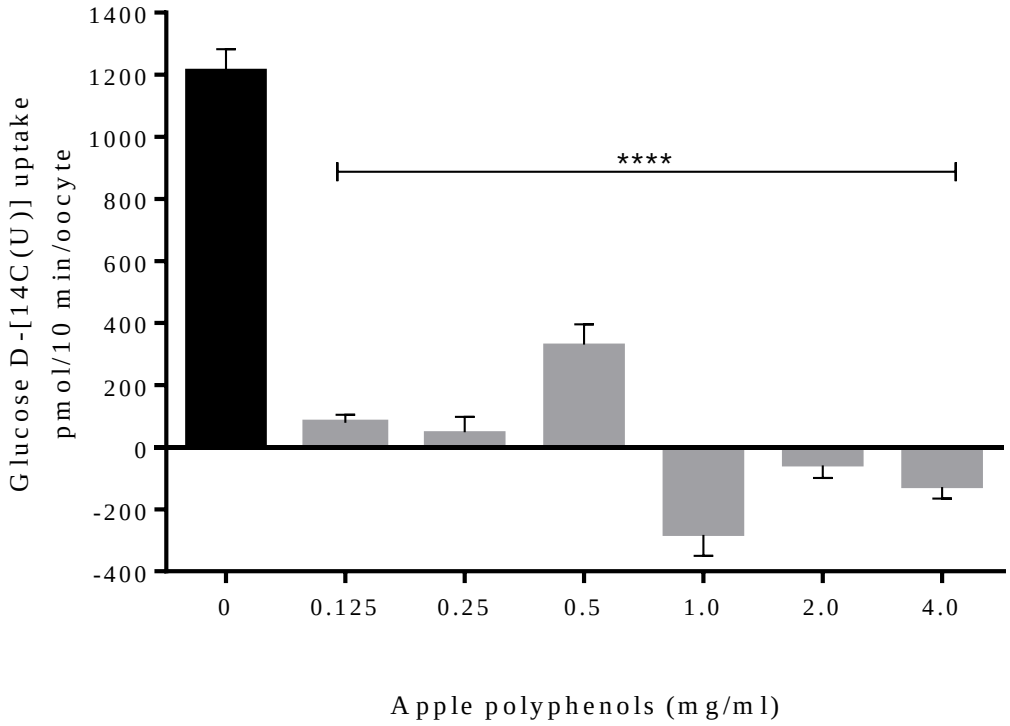


Figure 4.16 Effects of apple polyphenols (AE2) on glucose uptake on oocytes expressing rSGLT1

Oocytes injected to express rSGLT1 were treated with increasing concentrations of apple polyphenols. Values were corrected by subtracting sham oocytes uptake. Data are presented as mean ± SEM (n=3). One-way analysis of variance followed by multiple comparisons against control with Dunnett's adjustment ****P<0.0001 for the difference between 0 mg/ml and 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 mg/ml.

4.5 Discussion

As reported in our clinical trials (Chapter 3), the ingestion of blackcurrant and apple polyphenol-rich extracts decreased plasma glucose levels following starch and sucrose containing meals, and inhibited the secretion of insulin, GIP and to a lesser extent GLP-1. Evidence from cell studies suggests that the inhibition of small intestinal glucose uptake by polyphenols is a potential mechanism mediating these effects (Johnston *et al.*, 2005, Alzaid *et al.*, 2013). Therefore, the effects of anthocyanin-rich blackcurrant extract and polyphenol-rich apple extracts used in the clinical trials on glucose uptake were tested in Caco-2 cells, a well-established *in vitro* method of enterocytes in small intestine, and also in oocytes injected to express individual intestinal sugar transporters. Our i*n vitro* results demonstrated that the anthocyanin-rich blackcurrant extract (BE) and polyphenol-rich apple extracts (AE1 and AE2) significantly reduced total and GLUT-mediated glucose uptake into human Caco-2/TC7 cells and glucose uptake in *Xenopus laevis* oocytes injected to express solely SGLT1.

Our i*n vitro* results showed that blackcurrant extract (BE) and apple extracts (AE1 and AE2) significantly reduced glucose uptake into human Caco-2/TC7 cells by inhibition of glucose transporters but pure blackcurrant anthocyanins and anthocyanidins did not show an inhibitory effect. AE2 exerted the greatest effect, with an inhibition of 50% on total glucose uptake (likely to be both SGLT1- and GLUT-mediated) after exposure to 0.3 mg total polyphenols/ml, followed by BE with an inhibition of 50% by 0.5 mg/ml and AE1 with 30% inhibition after 0.3 mg/ml. GLUT-mediated glucose uptake (sodium-free conditions, effectively removing any transport by SGLT1) had a similar magnitude of inhibitory effect, with an inhibition of 46% by 0.3 mg/ml of AE2, followed by BE with an inhibition of 50% by 0.6 mg/ml and AE1 with 18 % inhibition after 0.3 mg/ml treatment. *In vivo* these concentrations of apple and blackcurrant polyphenols are achievable in the gut lumen after consumption of 2 medium apples (300 g, whole raw dessert apple, *Malus domestica*) and a portion/handful of fresh blackcurrant fruit (130 g, *Ribes nigrum* L.), respectively (Rothwell *et al.*, 2013). Comparison between results from total glucose uptake (SGLT1- and GLUT-mediated uptake) and GLUT-mediated glucose uptake suggest that inhibition of GLUT-mediated uptake by AE2 and BE was the main component in the attenuation of glucose uptake on the Caco-2 cells system. On the contrary, results from AE1 suggest that inhibition of SGLT1 transporter was a major component in the attenuation of glucose uptake by AE1

on the Caco-2 cells system, despite the fact that the phlorizin contents of both apple extracts were similar.

Inhibition of glucose uptake at concentrations BE 0.8 mg/ml and AE2 0.3 and 0.5 mg/ml are consistent with outcomes showed in GLU-BERRY and GLU-FRU studies, where a reduced postprandial glycaemia was observed after ingestion of the top dose test drink in GLU-BERRY (corresponding to estimated gut lumen concentrations of 0.8 mg blackcurrant polyphenols/ml) and the two tests drinks in GLU-FRU (corresponding to estimated gut lumen concentrations of 0.3 and 0.5 mg apple polyphenols/ml). Lower concentrations than those estimated in gut lumen after ingestion of effective doses on the human studies also inhibited glucose uptake in the Caco-2/TC7 system, although to a lesser extent. In the Caco-2 system concentrations equivalent to top doses of BE and AE2 test drinks inhibited total glucose uptake by more than 60%, while equivalent concentration of top dose test drink in GLU-APP (AE1 0.3 mg/ml) only inhibited total glucose uptake by 30%, this might explain the lack of effect on postprandial glycaemia by AE1 on the human study, and suggests that higher concentrations of AE1 may have been required to exert an inhibitory effect on postprandial glycaemia *in vivo*.

HPLC analysis of BE revealed high concentrations of anthocyanins, accounting for 49 % of the total polyphenols. A secondary hypothesis of this research was that anthocyanins were responsible for reduced glucose uptake into enterocytes, potentially by interaction with SGLT1 or GLUT2. Physiological concentrations of the anthocyanins; cyanidin-3-glucoside, cyanidin-3-rutinoside, delphinidin-3-glucoside and delphinidin-3-rutinoside and the anthocyanidins; cyanidin and delphinidin were tested. Anthocyanins and anthocyanidins did not decrease total glucose uptake in our Caco-2 cell model, this was an unexpected result since opposing findings were previously reported. Caco-2 cells treated with cyanidin, cyanidin-3-glucoside or cyanidin-3-rutinoside (each 0.1 mM) showed a decreased of ~20% on total and GLUT-mediated glucose uptake (Alzaid *et al.*, 2013). We did not find a significant inhibitory effect when testing cyanidin (0.18 and 0.10 mM), cyanidin-3-glucoside (0.03 and 0.10 mM) and cyanidin-3-rutinoside (0.15 and 0.10 mM) on total glucose uptake. We did not test specifically GLUT-mediated glucose uptake, but since total uptake was unaffected then it GLUT-mediated effects would also be expected to be nil. Differences in cell culture conditions might explain opposing results in inhibition of glucose uptake by anthocyanins, since 24 h incubation with serum-free media previous to transport assays,

as in Alzaid *et al.* study, could stress the cells and modify the functionality of the cell monolayer (Sambuy *et al.*, 2005). However, i*n vitro* studies have suggested the involvement of glucose transporters SGLT1 and GLUT2 in the absorption of cyanidin-3-glucoside (10 μM), since absorption into Caco-2 cells decreased when inhibitors phlorizin (10, 100 μM) and phloretin (1, 10, 100 μM) were added to uptake media (Zou *et al.*, 2014) which might represent a route for competitive inhibition of glucose uptake by anthocyanins. Therefore further studies testing higher concentrations of BE anthocyanins and anthocyanidins under sodium-dependent and sodium-independent conditions might help to identify a possible inhibitory mechanism, although the physiological relevance of higher concentrations would be debatable. Since in our *in vitro* studies anthocyanins did not inhibit glucose uptake, it is therefore possible that other polyphenol compounds contained in the BE were responsible for the inhibition of glucose uptake. We do not know the polyphenol composition of the remaining 51 % of BE extract, however analyses of the raw fruit have showed significant quantities of proanthocyanidins, and to a lesser degree, flavanols and phenolic acids (Rothwell *et al.*, 2013); individual compounds of the flavanol and phenolic acid subclasses have showed inhibitory effects on glucose uptake in Caco-2 cells (Manzano and Williamson, 2010, Johnston *et al.*, 2005, Dejiang *et al.*, 2011). Previous *in vitro* studies suggest that the type and degree of inhibition varies according to the presence or absence of a sugar moiety in the structure. There is some evidence that glucosides (e.g. flavonol glucosides) inhibit primarily SGLT1 transporters and aglycones (e.g. flavanols) regulate GLUT transporters (Kwon *et al.*, 2007), but further studies are required to confirm this. The combination of polyphenolics structures (aglycones and glucosides) that could potentially be in the blackcurrant extract may partially explain the marked inhibitory effect, since they may be acting through multiple mechanisms to inhibit glucose transport.

HPLC analysis of the apple extracts were performed by different companies but they showed similar concentrations of phlorizin, a well-known SGLT1 inhibitor, and differences in the proportions of other polyphenols such as quercetin, epicatechin and proanthocyanidins. When tested alone, phlorizin at 0.5 mM inhibited total glucose uptake by 23%. AE2 decreased total glucose uptake by 50 and 70% at 0.3 and 0.5 mg/ml concentrations, respectively, we estimated phlorizin concentrations in those samples of AE2 to be 0.05 and 0.1 mM, respectively, we therefore expect that lower

concentrations of phlorizin show lower inhibitory effects. Phlorizin was among the major components in both extracts and it is unlikely that the greater effect of AE2 on glucose inhibition was attributable to it. Other compounds are present and potentially, contributing to inhibition. Other polyphenols, such as phloretin and chlorogenic acid, contained in the both apple extracts but not measured in both, have showed inhibitory effects on glucose uptake in Caco-2 cells (Manzano and Williamson, 2010). An *in vitro* study using Caco-2 cells indicated an inhibition of 50% on total glucose uptake by phloretin 0.5 mM and by chlorogenic acid 1.3 mM (Manzano and Williamson, 2010). In our *in vitro* studies total and GLUT-mediated glucose uptake was inhibited in 22 and 14% by phloretin 0.5 mM, respectively. Concentration of phloretin in AE1 0.5 mg/ml was estimated to be 0.03 mM, although phloretin concentration was not specified for AE2, HPLC analysis suggested a concentration lower than 0.5 mM. Chlorogenic acid concentration in 0.5 mg/ml of AE2 was estimated to be 0.04 mM but the concentration was not available for AE1. Although concentrations of individual compounds in both apple extracts seem to be too low to exert a major percentage of inhibition separately, an additive effect might explain the inhibitory effect on glucose uptake since inhibition in Caco-2 cells has also been showed by several other polyphenols contained in the apple extracts, such as flavan-3-ols; (+)-catechin, (-)-epicatechin, epigallocatechin gallate, epigallocatechin and epicatechin gallate and flavonol quercetin (Johnston *et al.*, 2005, Dejiang *et al.*, 2011).

Limitations to the present study include the need to further test other individual metabolites, besides anthocyanins, contained in blackcurrant and apple extracts e.g. proanthocyanidins, flavan-3-ols, flavonols and phenolic acids. Moreover the differences between *in vivo* and *in vitro* conditions should be considered. Although the Caco-2 cell system is a widely accepted model of the small intestine, in the *in vitro* system there is not interaction of the metabolites with other tissues, enzymes or proteins that might modify their structure and therefore their bioavailability and mechanism of action. Additionally, the approach precludes any simulation of the conditions of continuous absorption and excretion of metabolites by the enterohepatic circulation in the small intestine, and therefore any findings are just an indication of potential effects *in vivo* and should be interpreted cautiously.

The identification of which specific transporter was blocked by the blackcurrant and apple extracts was not possible since Caco-2 cells/TC7 express different glucose transporters namely SGLT1, GLUT1, GLUT2, GLUT3 and the fructose transporter GLUT5. To study inhibition of individual sugar transporters we employed the *Xenopus* oocytes expression system in which individual transporters can be isolated. *Xenopus* oocytes have proved to be a reliable system to study glucose transporters activity as they exhibit low levels of endogenous glucose transport activity in shams (non-injected or water-injected) oocytes (Gould and Lienhard, 1989). Since human enterocytes under normal conditions only express the glucose transporters SGLT1 and GLUT2 and results using Caco-2 cells suggest that GLUT-mediated glucose uptake was the major component in the total glucose uptake, clones of rSGLT1 and hGLUT2 were tested in the oocyte system. Only rSGLT1 system showed consistent results in the validation assays, hGLUT2 system was still inconsistent (data not shown) by the time this document was written.

Our i*n vitro* results demonstrate that blackcurrant extract (BE) and apple extract (AE2) significantly reduced glucose uptake into *Xenopus laevis* oocytes by inhibition of SGLT1. Inhibitory effects were similar to findings using Caco-2 cells; in oocytes injected to express SGLT1, AE2 demonstrated the most effective inhibition compared to BE. The lowest concentration of apple extract tested, 0.125 mg polyphenols/ml, proved to inhibit glucose uptake by 90% while comparable BE concentration 0.12 mg polyphenols/ml, showed inhibition of 50%. These results are in agreement with previous reports showing 50% glucose uptake inhibition by 0.24 μg of phlorizin-rich apple extract per mL and by 0.3 mg of quercetin-rich onion extract per mL, via inhibition of hSGLT1 (Schulze *et al.*, 2014, Schulze *et al.*, 2015). BE showed a dose-response inhibition; to our knowledge we are the first showing decreased glucose uptake by a berry extract in oocytes expressing rSGLT1. Individual anthocyanins were not tested in the present study, however assays testing supra-physiological levels of anthocyanins pelargonidin and pelargonidin-3-glucoside (which were not detected in BE) showed 50% inhibition of hSGLT1 by 1.34 and 2.47 mM of pelargonidin and pelargonidin-3-glucoside, respectively (Kottra and Daniel, 2007). More studies testing physiological concentrations of individual anthocyanins contained in the blackcurrant extract are needed to elucidate the possible effect on SGLT1, although the magnitude of the relative contribution to overall inhibition of glucose transport *in vivo* is likely to be

insignificant when taken in the context of the Caco-2 cell experimental results reported here.

Apple extract (AE2) showed a greater inhibitory effect than BE on SGLT1-mediated glucose transport in oocytes, but the marked inhibition at the lowest dose tested meant that we were unable to demonstrate a dose-response inhibition. More assays testing lower concentrations of AE2 are needed in order to identify the IC_{50} value. SGLT1 inhibition by several polyphenols contained in the apple extract has been demonstrated, e.g. competitive inhibition by the glycoside phlorizin (Kobayashi *et al.*, 2000, Kottra and Daniel, 2007, Schulze *et al.*, 2014) and non-competitive inhibition by the aglycone phloretin (Hirayama *et al.*, 2001, Schulze, 2014). When tested individually phlorizin and phloretin 0.5 mM inhibited rSGLT1-mediated glucose uptake by 85 and 59%, respectively. AE2 0.125 mg/ml inhibited rSGLT1 glucose uptake by 90%, concentration of phlorizin contained was 0.02 mM, even though phloretin concentration in AE2 was not specified, HPLC analysis suggests a concentration much lower than 0.5 mM, therefore is it unlikely that the additive effects of phlorizin and phloretin alone accounted for the total inhibition showed by AE2. It might be possible that other components of the apple extract such as quercetin (0.02 mM in 0.5 mg/ml of AE2) and proanthocyanidins were also bioactive in this respect; flavonols quercetin (IC_{50}= 0.62 mM) and quercetin-4-O-glucoside (IC_{50}= 0.17 mM) have previously been shown to inhibit hSGLT1 (Kottra and Daniel, 2007, Schulze *et al.*, 2015). However other polyphenols contained in the AE2, such as chlorogenic acid (0.04 mM in 0.5 mg/ml of AE2) have shown no inhibitory effects on SGLT1 at a higher concentration (0.1 mM) (Schulze *et al.*, 2014).

SGLT1 is also expressed in enteroendocrine cells of the small intestine and has been shown to be closely related with the expression of GIP and GLP-1 using individual enteroendocrine cells of murine jejunal crypts. Studies in *Sglt1*$^{-/-}$ (knocked out) mice have shown the essential role of SGLT1 as a glucose sensor for secretion of gut incretins GIP and GLP-1 and upregulation of GLUT2 (Gorboulev *et al.*, 2012). Moreover, a separate study in mice reported inhibited glucose induced SGLT1-mediated GIP secretion by apple polyphenol phlorizin (Moriya *et al.*, 2009). Our clinical studies showed a decreased secretion of GIP after BE and AE2 treatments. The oocyte SGLT1 inhibition studies presented here open up the possibility that inhibition of glucose-induced GIP secretion observed in vivo may have been directly mediated via blockade

of SGLT1 expressed by GIP-secreting K cells; this theory may be investigated in future research using cultured STC-1 cells, a model for GIP-secreting enteroendocrine cells.

The blackcurrant and apple extracts in the current study contained a wide range of individual compounds with potential inhibition activity of both SGLT1 and GLUTs transporters, and a synergistic/additive effect might be responsible for the decreased on glucose uptake in Caco-2/TC7 cells and *Xenopus* oocytes systems. However the presence of high molecular weight polymers such as proanthocyanidins (PA), might also contributed to the highly significant inhibition on glucose uptake. The polymeric proanthocyanidins are components of both blackcurrant and apple polyphenol profiles. They are estimated to constitute 25 and 50% of the BE and AE2 polyphenols, respectively and consisting in blackcurrant, of dimers (2%), oligomers 3-10 mers (15%) and polymers >10 mers (82%) and in apple, of dimers (11%), oligomers 3-10 mers (52%) and polymers >10 mers (30%) (Rothwell *et al.*, 2013). Proanthocyanidins (PA) are less able to permeate into intestinal cells, and show a high degree of interaction with the plasma membrane. Probable factors affecting absorption are size and polymerisation degree, hydrophilic characteristic and affinity for luminal proteins (Deprez *et al.*, 2001). The absorption of PA tends to decrease when polymerisation increase, PA dimers and oligomers (2-5 mers) have demonstrated being absorbable but PA polymers (>5 mers) has showed nil permeation into intestinal cells in studies with animals and humans (Neilson *et al.*, 2016). High molecular weight polyphenolic molecules such as PA, with a size up to 10 kDa (Yanagida *et al.*, 2003), a high degree of polymerisation and hydrophilic characteristics, may interact by hydrogen bonding with the polar head groups of the phospholipids in the outer leaflet of the plasma membrane coating the surface and covering proteins inserted in the plasma membrane (Tarahovsky, 2008). Glucose transporters SGLT1 and GLUT2 are transmembrane protein with size range 69-79 kDa for the former (Wright *et al.*, 2011) and 69 kDa for the latter. Considering the possible quantity of polymers polyphenols present in the extracts, it might possible that a percentage of the glucose uptake inhibition correspond to a hindering of the glucose transporters by PA complexes rather than to a specific inhibition of the glucose transporters by monomeric polyphenols. Moreover structural studies have shown that the side chains of SGLT1 (C-13 terminal loop) interact with lipid bilayers (Raja and Kinne, 2005) therefore it is possible that polyphenol-phospholipid interactions interfere with SGLT1 conformational changes needed for substrate binding, resulting in

inhibition of glucose transport. A possible interaction between PA and integral membrane proteins by covalent and non-covalent bonds (Kroll *et al.*, 2005) might also explain the blocking of glucose transporter structures by a specific or/and non-specific inhibition (Blade *et al.*, 2016).

Our results suggest that polyphenols contained in the blackcurrant and apple extracts used in our clinical trials inhibited glucose transporters; further investigation is necessary in order to identify the individual compounds of blackcurrant and apple extracts that played a significant role in the inhibition of glucose uptake. Testing physiological concentrations of different fractions of the extracts such as PA on Caco-2 cells and oocytes, and individual compounds such as anthocyanins in oocytes, might help to identify the bioactive compounds and the glucose transporters involved. *In vitro* studies using oocytes expressing GLUT2 might help to identify if the polyphenols had an equal or greater effect on this transporter relative to SGLT1, in agreement with the Caco-2 cell experiments. *In vitro* enzymatic assays testing the extracts will be useful to identify the effect of blackcurrant and apple polyphenols on digestive enzymes, such as α-amylases and α-glucosidases, which play an essential role in the metabolism of carbohydrates.

4.5.1 Conclusion

Results from our clinical trials in humans have indicated that a moderate dose (1200 mg) of blackcurrant polyphenols and/or apple polyphenols and a combination of blackcurrant and apple polyphenols (1200 mg + 600 mg) significantly lowered postprandial glycaemia, insulinaemia and gut hormone GIP levels in healthy volunteers. Although the mechanism of action still needs further exploration, our *in vitro* results suggest that the polyphenols contained in blackcurrant and apple extracts may reduce postprandial glycaemia by inhibiting glucose transporters since physiological concentrations of the extracts decreased effectively total (SGLT1- and GLUT-mediated) and GLUT-mediated glucose uptake in Caco-2/TC7 cells and inhibited glucose uptake via blocking of rSGLT1 expressed in oocytes. Regular dietary consumption of fresh blackcurrant and apple may improve glycaemia response by limiting or delaying glucose absorption in the intestine, and may also reduce postprandial insulinaemia by decreasing glucose-induced SGLT1-mediated GIP secretion. Future work could determine the relative impact of inhibition of both digestive enzyme action and glucose transporters, which would help inform developments in pharmaceutical science aimed at lowering postprandial hyperglycaemia in type 2 diabetes.

Chapter 5

General discussion and conclusion

The overarching thesis of this research is that regular dietary intakes of blackcurrant and apple polyphenols may help reduce risk of cardio-metabolic diseases by lowering postprandial blood glucose concentrations. The results presented here show that: 1) urinary biomarkers of dietary intakes of polyphenols contained in apples (phloretin) and blackcurrants (e.g. gallic acid) are increased in a population who are following UK dietary guidelines and report higher fruits and vegetables intake in a food frequency questionnaire; 2) consuming blackcurrant and apple extracts immediately before a starch and sucrose containing meal suppresses the rise in blood glucose concentrations; 3) using an *in vitro* model, blackcurrant and apple extracts inhibit glucose transport into intestinal cells. Linking these findings together, it might be suggested that adherence to fruits and vegetables dietary guidelines may help regulate blood glucose levels if consumed with a high-carbohydrate meal, and this is at least partly mediated by delaying the absorption of glucose.

The implications of this research are that dietary recommendations could emphasise polyphenol-rich fruits such as berries and apples in order to increase the polyphenol consumption in the population. Chronic consumption of polyphenol-rich fruits may have a cumulative effect on metabolic disease risk through repeated episodes of lowering postprandial glycaemia, in addition to any beneficial cardio-metabolic effects of increased circulating polyphenol metabolites in the later phase and beyond of the postprandial period through interaction with endothelial cells, pancreatic beta cells, adipocytes, liver cells and any other tissues relevant to the pathogenesis of type 2 diabetes and cardiovascular disease (Hanhineva *et al.*, 2010). The marked effect on plasma glucose, insulin and GIP concentrations showed by the extracts used in the clinical trials administering a starch and sucrose challenge indicate that polyphenols are more effective inhibiting postprandial glycaemia when digestives enzymes are involved in the breakdown of carbohydrates than when intestinal glucose transporter are only responsible for glucose absorption (glucose load). Diverse phenolic compounds contained in the blackcurrant and apple extracts are potentially responsible for the beneficial effects, e.g. proanthocyanidins, anthocyanins, phenolic acids, flavonols, flavan-3-ols. Evidence from *in vitro*/animal models suggests that GIP secretion appears to act as sensitive marker of glucose absorption linked to the SGLT1 transporter (Gorboulev *et al.*, 2012; Röder *et al.*, 2014) and the marked suppression of postprandial GIP response may indicate a possible direct inhibition of the glucose transporter SGLT1 in enteroendocrine cells. The type of polyphenols ingested suggests differences on

glucose response; blackcurrant polyphenols (1600 mg, GLU-BERRY) had a less marked effect on glucose homeostasis than a smaller amount of apple polyphenols (1200 mg, GLU-FRU). It seems likely that differences in the extracts profile have had an influence on the results. Apple extract was particularly rich in proanthocyanidins, flavan-3-ols and phenolic acids besides phlorizin, which have proved to be effective inhibiting digestive enzyme activity and glucose transporters GLUT2 and SGLT1. Results from mechanistic studies testing blackcurrant and apple extracts are congruent with results from clinical trials. Apple extract inhibited glucose uptake in a stronger manner than blackcurrant extract, therefore the *in vitro* Caco-2 cells system could be used as prediction model when establishing doses for *in vivo* studies. Mechanisms of inhibition are complex to elucidate; further analysis, to test individual compounds besides anthocyanins, are needed. The mixture of polyphenols contained in blackcurrant and apple extracts include aglycones, glycosides and low and high molecular weight, all variables to take into account when testing inhibitory effects.

In the dietary analysis of the CRESSIDA study participants (**Chapter 2**) it was found that adherence to UK dietary guidelines led to an increased self-reported intake of polyphenols associated with reduced risk of T2D such as anthocyanins (Wedick *et al.*, 2012). Increases in phenolic acids, isoflavones and lignans were also observed. Phenolic acids have been associated with improvement of endothelial function (Del Rio *et al.*, 2013). Intake of two classes of phytoestrogens, isoflavones and lignans, are closely related with intake of whole grain products, which are indicators of a healthier diet pattern, associated with reduced risk of T2D (Talaei and Pan, 2015). Although the main sources of polyphenols in the CRESSIDA population were non-alcoholic beverages such as tea and coffee, adherence to UK dietary guidelines showed an increased consumption of anthocyanin-rich fruits such as berries, phenolic acid-rich fruits such as apples, isoflavone-rich food such as soy-derived beverages and lignan-rich food such as nuts and linseed. The current UK dietary guidelines recommend an intake of $\geq$ 5 portions of fruit and vegetables per day (400 g/d), and higher consumption of fruits and vegetables by themselves have been associated with reduced risk of T2D (Wang *et al.*, 2016, Mursu *et al.*, 2014), and greater consumption of specific fruits, such as blueberries, grapes and apples and green leafy vegetables has also been associated with reduced risk of T2D (Muraki *et al.*, 2013, Cooper *et al.*, 2012). It could be argued that an increase in the recommended intake of F&V in combination with more focused advice including the recommendation of higher intakes of berries, apples and other

polyphenol-rich fruit/vegetable, instead of general recommendation of fruits and vegetables, would be helpful in some populations. A similar approach have been adopted by Finland where berries intake is recommended as separate item from general fruits and the recommended intake of vegetables, berries and fruit was raised to 500 g/d instead of 400 g/d (5 portions/d). The Mediterranean diet (Greece, Spain) has elevated recommend intakes of F&V, 9 servings per day (~720 g) (USDA, 2016). China has higher recommended intakes of F&V (500-900 g/d) and has also established a system of dietary reference intakes (DRIs) values for non-essential bioactive compounds including polyphenols, such as anthocyanin, resveratrol, quercetin, proanthocyanidins, chlorogenic acid and isoflavones (Lupton *et al.*, 2014). Geographical and seasonal availability of fruits such as berries must be considered if specific recommendations are intended. Therefore it might be more convenient if recommendations take into account foods that are broadly available and with combined high concentrations of other diverse polyphenols associated with reduced risk of T2D, such as proanthocyanidins (Zamora-Ros *et al.*, 2013), e.g. foods with high concentration of proanthocyanidins dimers/trimers plus anthocyanins (plum, grape, strawberry, redcurrant, blackberry and sweet cherry), or foods high in proanthocyanidins dimers/trimers plus flavan-3-ols, (apple, peach). Although all dietary analysis *per se* has significant methodological limitations and biomarkers of dietary intake provide a more objective association analysis, nevertheless evidence suggests that regular intake of specific polyphenol-rich fruit and vegetables decrease the risk of T2D.

Results presented in **Chapter 3** showed that polyphenol-rich blackcurrant and apple extracts attenuated postprandial hyperglycaemia and inhibited the secretion of insulin and incretins. It is apparent from these studies that there is a role for blackcurrant and apple polyphenols in regulating digestion and absorption of carbohydrates. Gastrointestinal interactions, in combination with post-absorptive effects of blackcurrant and apple polyphenols, might be expected to result in a sum normalisation of the postprandial glucose curve if such fruits were habitually consumed with meals. However, considerations about the food matrix must be taken into account since blackcurrant and apple fresh fruit contained other components that might influence the postprandial response, such as fibre which might delay the rate of glucose absorption by slowing down gastric emptying thereby slowing down the digestion process and increasing satiety, and proteins that may bind to the proanthocyanins thus reducing their availability for interaction with intestinal enzymes. Plant cell walls might also act as a

mechanical barrier to digestion of other components contained in fruits and interaction of non-starch polysaccharides and non-digestible oligosaccharides with microbiota in colon might exert a subsequent action on satiety as well (Blaak *et al.*, 2012). On the other hand, fruits also contain fructose, high intakes of which have been related with increased liver fat, hepatic insulin resistance and increased serum uric acid level, although there is still disagreement about the clinical effects of fructose when consumed in normal levels (Rippe and Marcos, 2016, Johnston *et al.*, 2013), and the total amount of fructose (as well as glucose) in the diet must be considered when targeting the control of postprandial glycaemia, particularly in developing food/drink products containing fruit syrups/nectars/concentrates high in polyphenols but not contained within cell walls.

Besides the acute effect on postprandial glycaemia shown by blackcurrant and apple polyphenols in human studies, a number of animal studies have demonstrated acute and chronic inhibitory effects of berry anthocyanins and apple polyphenols on weight gain, fasting glucose and increases in insulin sensitivity. Mice consuming a very high-fat diet were changed to a low-fat diet supplemented with blueberry concentrate (providing an average of 4.4 mg blueberry anthocyanins/d), which resulted in a reduction in weight gain and increase in glucose tolerance compared to the blueberry-free low-fat and high-fat controls (Roopchand *et al.*, 2013). Purified blueberry anthocyanins (0.49 mg/d) reduced fasting glucose levels in mice receiving a high-fat diet to levels comparable with a low-fat diet, and also reduced percentage body fat and body weight (Prior *et al.*, 2010, Jayaprakasam *et al.*, 2006), and pure cyanidin-3-glucoside (C3G) supplementation resulted in markedly lower fasting blood glucose concentrations after 3 weeks of high-fat diet compared to high-fat diet with no supplementation, as well as in diabetic mice consuming regular chow (Guo *et al.*, 2012). Diabetic KK-A^y mice subjected to 5-week daily administration of concentrated bilberry extract (27 g/kg diet) showed a marked reduction in fasting plasma glucose and improved insulin sensitivity (Takikawa *et al.*, 2010). Similar results have been shown by apple extracts and apple polyphenols; acute administration of apple extract (150 mg/kg body weight) decreased glycaemic response (iAUC) following an oral load of maltodextrin in OZR mice, an insulin resistant model (Manzano *et al.*, 2016). The effect of the apple extract was further tested with a second meal (maltodextrin) 5 h after the administration of the apple extract; the group supplemented with apple extract showed a 20% decrease in plasma glucose AUC when compared to the control group. In the same study chronic

administration of the apple extract improved glycaemic and insulinaemic responses in the OZR group supplemented with 3 g of apple extract/kg diet for four weeks. In a separate study a 10-day treatment with intraperitoneal injection of the apple polyphenol, quercetin (10/15 mg per kg of body weight) decreased plasma glucose levels in streptozocin-induced diabetic rats in a dose-response manner following a glucose tolerance test (Vessal *et al.*, 2003). Animal studies have showed favourable effects of berry and apple polyphenols on glycaemic control during fasting and postprandial states in mice. The evidence as a whole suggests that berry anthocyanins and apple polyphenols may have potentially preventative effects on glycaemia and weight gain.

In our *in vitro* assays (**Chapter 4**), physiological concentrations of blackcurrant and apple polyphenols significantly inhibited glucose uptake in *in vitro* models of small intestinal glucose absorption, via blocking glucose transporters SGLT1 and GLUTs. A number of *in vitro* studies have suggested that metabolism of blackcurrant and apple polyphenols might play an important role in the beneficial effect on postprandial glycaemia in human studies. Anthocyanin metabolites and berry and apple flavonoids have been shown to influence muscular glucose uptake, insulin signalling, and adipocytokine expression in *in vitro* studies in human and rat cells and in animal studies (Babu *et al.*, 2013). Both berry and apple polyphenols and metabolites activate proteins involved in glucose uptake, such as AMP protein kinase (AMPK) and phosphoinositide 3-kinase (PI3K) in skeletal muscle cells and mesenteric adipose and liver tissues. AMPK is involved in the secretion and membrane translocation of the glucose transporter GLUT4 in muscle cells and its activation in liver tissue might result in the down-regulation of glucose-synthesising genes. Berry anthocyanins and apple polyphenols have also been able to increase transcription of the peroxisome proliferator-activated receptor (PPARs) involved in the expression of GLUT4 and adipocytokines, both effects related to increased insulin sensitivity in muscle cells and liver (Scazzocchio *et al.*, 2015, Scazzocchio *et al.*, 2011, Tsuda *et al.*, 2004, Takikawa *et al.*, 2010, Manzano *et al.*, 2016, Kim *et al.*, 2016, Wang *et al.*, 2014, Santangelo *et al.*, 2016). Altogether, these data suggest that in addition to influencing carbohydrate digestion and absorption, circulating blackcurrant and apple polyphenols metabolites may act to increase insulin sensitivity. Decreased glucose absorption and improved insulin sensitivity in hepatic and skeletal muscle tissue are targeting points in the pharmacological treatment of T2D. For example AMPK acts as sensor of cellular energy status and when activated stimulates processes to generate ATP, such as the

translocation of GLUT4 transporters in order to increase cellular influx of glucose (Hardie, 2008); this mechanism of action is actually exerted by some of the drugs most commonly used in diabetes management. Nowadays treatment for T2D includes a range of medications with different targets and mechanism of action, such as metformin to decrease glucose production by liver and increase insulin sensitivity (by indirect activation of AMPK), sulphonylureas and prandial glucose regulators to stimulate insulin secretion by pancreatic β-cells and sensitise β-cells to glucose, acarbose to inhibit α-glucosidase activity, thiazolidinedione to reduce insulin resistance and improve insulin sensitivity (by direct and indirect activation of PPARs and AMPK, respectively), incretin mimetics/GLP-1 analogues and DPP-4 inhibitors to increase insulin secretion and slow down digestion and gastric emptying (also AMPK activators) and SGLT2 inhibitors to reduce glucose absorption in kidneys, therefore reduce blood glucose (as review in Coughlan *et al.*, 2014 and Diabetes UK, 2016). In summary evidence from *in vitro* and animal studies suggests that berry and apple polyphenols may have potentially use as hypoglycaemic treatment in T2D.

5.1 Future work

Further randomised controlled clinical trials to identify the minimal effective dose on postprandial glycaemia by apple polyphenols are planned. Including quantification of polyphenol metabolites in biological samples, such as plasma and urine, would be helpful to identify the absorption and metabolism of polyphenols consumed in the test drink/meal. Investigating the durability of the effect of polyphenols on postprandial glycaemia with a second high-carbohydrate meal would help in formulating dietary advice on how to optimise normal glucose homeostasis by consumption of apples and berries. Quantification of other parameters such as glucose concentrations in urine might help to detect other effects like inhibition of SGLT2 in the kidney, reducing reabsorption of glucose back into the circulation and increasing urinary glucose output, which might be of relevance in the management of T2D. Collaboration with the food industry to produce and test functional foods supplemented with the blackcurrant and/or apple extracts will help to the development of commercially available and palatable beverages and foodstuffs. Further mechanistic insights might be made using additional *in vitro* studies focussing on structural relationships between individual blackcurrant and apple polyphenols and glucose transport GLUT2 and digestive enzymes which may highlight possible synergistic effects of different polyphenols, increasing the potential to exploit these mechanisms in the improvement of postprandial hyperglycaemia. The study of potential effects of blackcurrant and apple polyphenols and their metabolites in different *in vitro* models such as enteroendocrine and muscle cells, might be helpful to understanding the role played out by polyphenols in the reduction of postprandial glycaemia observed in the clinical trials.

5.2 Conclusion

Blackcurrant and apple polyphenols and metabolites decrease postprandial glycaemia, insulin secretion and GIP secretion *in vivo* and inhibit glucose absorption *in vitro*. The potential beneficial effects exerted by blackcurrant and apple polyphenols mimic several mechanisms targeted by T2D treatment drugs, such as inhibition of α-glucosidase activity, activation of AMPK and PPARs and inhibition of glucose transporters in small intestine (SGLT1 and GLUT2) and kidney (SGLT2). Therefore the habitual consumption of such fruits in a normal diet might be considered as hypoglycaemic treatment in pre-diabetic stages (IFG and IGT) and T2D, as a replacement or complement to pharmacological treatment, with the additional benefits of minimal side effects and representing a more sustainable economic strategy. The physiological concentrations proven to be effective in the human studies and in *in vitro* assays might represent challenging doses to be consumed immediately before a high-carbohydrate meal in their fruit form; however, the possibility remains that lower amounts, consumed on a more frequent basis, may exert similar results on the postprandial glucose response. In conclusion, consuming more apples and berries (or foods/beverages fortified with extracts from these fruits) presents an efficacious option for lowering postprandial glycaemia, but is somewhat limited in what this could achieve in terms of reducing risk of T2D. With obesity being the major risk factor for T2D, combined approaches with the potential for population-level impact could be considered to target healthy body weight maintenance and glucose homeostasis simultaneously. For example, food companies could add apple/berry extracts as functional ingredients to high-sugar/starch-containing products, in combination with measures to reduce glycaemic loads/energy contents, and to increase the satiating capacity of the same products. The novel findings presented in this thesis show that advice to consume more fruits and vegetables can effectively increase intakes of specific polyphenols that have been associated with reduced risk of cardio-metabolic diseases, and provides robust mechanistic evidence for mechanisms whereby non-nutrient bioactives derived from fruits might regulate glucose homeostasis.

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Appendices

Appendix 1 HPLC analysis of the apple extract used in the GLU-APP study

rssl

Certificate of Analysis

Mr Paul Jones
Lucozade Ribena Suntory Ltd
Royal Forest Factory
Rock Lane
Coleford
Gloustershire
GL16 8JB

Report No: P14-05628
Purchase Order: (None Supplied)
Date Received: 23rd July 2014
Date Started: 20th August 2014

BN-16412: Project: TSB

Sample Code: **P14-05628-1** Your Refs: BN-16412 ADC-04222-302213

Method	Analysis	Result	Units
	Phloretin	110.0	mg/100g
	Phloridizin	4992	mg/100g
TM-160	Quercetin	103.2	mg/100g
TM-161	Gallic Acid	<30	mg/100g
TM-161	Gallocatechin	<1	mg/100g
TM-161	Theobromine	<15	mg/100g
TM-161	Epigallocatechin	5467	mg/100g
TM-161	Catechin	28479	mg/100g
TM-161	Caffeine	<50	mg/100g
TM-161	Epicatechin	12010	mg/100g
TM-161	Epigallocatechin gallate	<10	mg/100g
TM-161	Gallocatechin gallate	<100	mg/100g
TM-161	Epicatechin gallate	182	mg/100g
TM-228	Total Polyphenols (as Gallic Acid Equivalent)	73	g/100g
TM-336	Monomer	31.9	%
TM-336	Dimer	28.3	%
TM-336	Trimer	14.7	%
TM-336	Tetramer	6.5	%
TM-336	Pentamer	6.5	%
TM-336	Hexamer	4.5	%
TM-336	Heptamer	2.8	%
TM-336	Octamer	1.4	%
TM-336	Nonamer	0.7	%
TM-336	Decamer	0.3	%

Science With Service

Approved By:
Rachel Reid
Senior Scientist I
(Functional Ingredients)
21 August 2014

READING SCIENTIFIC SERVICES LTD
Reading Science Centre, Whiteknights Campus
Pepper Lane, Reading, RG6 6LA
Tel: +44 (0)118 918 4000
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email: enquiries@rssl.com
web: www.rssl.com

Appendix 1 continued

Certificate of Analysis

Mr Paul Jones
Lucozade Ribena Suntory Ltd
Royal Forest Factory
Rock Lane
Coleford
Gloustershire
GL16 8JB

Report No: P14-05628
Purchase Order: (None Supplied)
Date Received: 23rd July 2014
Date Started: 20th August 2014

BN-16412: Project: TSB

Sample Code: **P14-05628-1** Your Refs: BN-16412 ADC-04222-302213

Method	Analysis	Result	Units

The procyanidin results are a percentage distribution calculated from the peak areas. The results could not be quantified as the standard used for analysis is an in house standard generated from cocoa beans.

Science With Service

Approved By:
Rachel Reid
Senior Scientist I
(Functional Ingredients)
21 August 2014

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web: www.rssl.com

Appendix 2 Advertisement for GLU-BERRY study

KING'S College LONDON

VOLUNTEERS WANTED

FOR NUTRITION RESEARCH

We are running a study (The GLU-BERRY 1 study) to look at the effects of a blackcurrant extract on your health.

We are investigating whether a substance found in blackcurrants may decrease the risk of diabetes by reducing the levels of blood sugars after a high-carbohydrate meal.

We are looking for:

- Men aged 20-60 years
- Women aged 45-60 years (postmenopausal)
- Able to attend King's College London, near Waterloo Station for 4 visits

You will be reimbursed for participation in the study and travel expenses

Please contact Leanne Smith on 020 7848 4356 or e-mail leanne.smith@kcl.ac.uk

REC ref. BDM/11/12-88

Appendix 3 Advertisement for GLU-APP study

REC ref. BDM/11/12-88

KING'S College LONDON

Want to know more about how apples could improve your health?

If you are healthy and a non-smoker, you may be able to help us. We need men aged 20-60 y and postmenopausal women aged 45-60 y.

The GLU-APP study aims to explore the effects of consuming apple extracts on levels of blood sugar after a meal. Participating will give you information on your blood fats and glucose levels, body composition and blood pressure. You will be compensated for your time. If you would like to know more, please contact Leanne by e-mail or phone:

leanne.smith@kcl.ac.uk
Tel: 020 7848 4356

Leanne.smith@kcl.ac.uk Tel:020 7848 4356
Leanne.smith@kcl.ac.uk Tel:020 7848 4356
Leanne.smith@kcl.ac.uk Tel:020 7848 4356
Leanne.smith@kcl.ac.uk Tel:020 7848 4356
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Leanne.smith@kcl.ac.uk Tel:020 7848 4356
Leanne.smith@kcl.ac.uk Tel:020 7848 4356
Leanne.smith@kcl.ac.uk Tel:020 7848 4356

Appendix 4 Advertisement for GLU-FRU study

REC ref. BDM/14/15-10

KING'S College LONDON
University of London

VOLUNTEERS NEEDED

If you are **healthy and a non-smoker**, you may be able to help us.
We need men and postmenopausal women aged 20-60 y.

The GLU-FRU study aims to explore the effects of consuming blackcurrant and apple extracts on levels of blood sugar after a meal. Participating will give you information on your body composition, blood pressure, vascular function, and glucose and insulin levels. You will be compensated for your time. If you would like to know more, please contact Monica Castro, Stephanie Stone or Jonathan Mok by e-mail or phone:

glu-frustudy@kcl.ac.uk
Tel: 020 7848 4162, M: 07526 787103

glu-frustudy@kcl.ac.uk T: 020 7848 4162 M: 07526 787103
glu-frustudy@kcl.ac.uk T: 020 7848 4162 M: 07526 787103
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Appendix 5 Information sheet for participants

Version Number 20/08/2014

KING'S College LONDON

INFORMATION SHEET FOR PARTICIPANTS

***BDM RESC Protocol Number* BDM/14/15-10**

YOU WILL BE GIVEN A COPY OF THIS INFORMATION SHEET

THE ACUTE EFFECTS OF BLACKCURRANT AND APPLE EXTRACTS ON POSTPRANDIAL GLYCAEMIA: A RANDOMISED CONTROLLED TRIAL. THE GLU-FRU STUDY

We would like to invite you to participate in this original research project undertaken as part of a PhD programme. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

What is the purpose of this study?
This research project is a dietary intervention study to investigate effects of natural plants constituents found in high amounts in fruit, called polyphenols, on the rise in blood sugar (glucose) that occurs following a meal. The purpose of this study is to test whether a combination of blackcurrant and apple extract can augment the effects of an apple extract in reducing the levels of blood glucose during a 2-hour period after a carbohydrate-containing meal, and to test if the combination of blackcurrant and apple extract improves the functioning of your blood vessels.

Why have I been chosen?
You have been contacted as you have expressed an interest in our research. In order to participate in this study you need to be able to say 'Yes' to the following:

- I am male and aged between 20 and 60 years old
- **OR** I am female and aged between 20 and 60 years old, and I am postmenopausal (my menstrual periods stopped at least 1 year ago, and I am not pregnant nor intending to become pregnant, nor breastfeeding)
- I do not smoke and have not recently given up smoking (within the last 6 months)
- I have never had a heart attack, stroke, angina, thrombosis, liver or kidney diseases, diabetes, chronic gastrointestinal disorder or cancer
- I do not have phenylketonuria (PKU) - *this is a rare genetic metabolic disorder which prevents the breakdown of an amino acid, phenylalanine and is apparent from early infancy.*
- I do not take medication to lower blood fats (e.g. statins, fibrates) or to stabilise blood glucose (e.g. acarbose, metformin or sulfonylureas)
- I do not have a history of excess alcohol intake or substance abuse
- I do not have food intolerances, allergies or hypersensitivity
- I am not already participating in a clinical trial
- I am prepared to follow dietary instructions before and during the study
- I have not recently donated blood (within the last 3 months)
- I have not recently lost more than 3 kg/7 lb (in the last 2 months)

What will happen to me if I take part?
If you would like to participate you would first need to complete a screening questionnaire with us over the telephone, in person or via email (approx. 15 mins), after which potentially eligible volunteers will be

Appendix 5 continued

invited to attend a clinic screening appointment (approx. 45 mins) in the Metabolic Research Unit on the 4th Floor, Corridor A, Franklin-Wilkins Building, 150 Stamford Street, SE1 9NH.

Summary of screening visit:

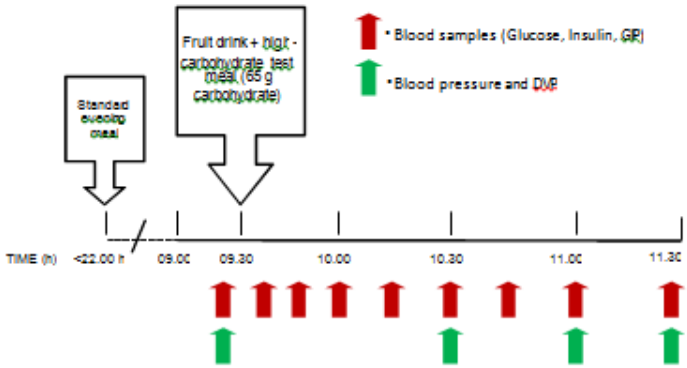
1) **You should avoid eating or drinking anything, except water, from after 20.00 h the previous night.**
2) **The visit will last approximately 45 min.**
3) **We will give you a copy of this information sheet, explain to you all the details of the study and answer any questions you have. If you are still happy to take part in the study, you will be asked to sign a consent form.**
4) **We shall ask you questions about your medical history, your food habits and measure your weight, height, percentage body fat, blood pressure and waist and hip circumference**
5) **We will need to take a small venous blood sample to extract capillary blood (16.5 ml, about 3 teaspoons) to check that your blood biochemistry is normal.**
6) **Then you will be provided with breakfast.**

The results of the screening blood test will be provided within 2 weeks. If any abnormal results are found we will offer to provide you with a letter for your GP. If, after screening, you are discovered to be unsuitable for the study your data will be destroyed.

If you are eligible for the study you will attend the Metabolic Research Unit at the Franklin-Wilkins Building for three study visits. Prior to the first visit you will be given a "Food and Drink Diary" in which we would like you to record everything that you eat and drink for 4 days. During the study day frequent blood samples will be taken, and measurements of blood pressure and blood vessel function by measuring changes in your pulse using a probe that clips on the finger (digital volume pulse; DVP) will be made. You will consume the fruit drink following by a high carbohydrate meal (white bread with apricot jam) after the first blood samples and measurements have been made. On each of these study visits the fruit drink will contain, blackcurrant and apple extract, apple extract or will be a placebo drink (containing no fruit extract). This visit takes approximately 3 h, including time to consume lunch afterwards. The total time you will be participating in this dietary intervention study will be at least 4 weeks, and a maximum of 9 weeks, depending on when the study visits are scheduled.

The overall study is shown in a diagram below:

Appendix 5 continued



Appendix 5 continued

Summary of study day visit:

1) Following screening, if your results comply with the study inclusion criteria you will be invited to attend the Metabolic Research Unit in the Franklin-Wilkins Building on 3 further occasions at least 7 days apart; each of these visits will take approximately 3 h each.
2) We will ask you to avoid fatty foods, oily fish, high-polyphenol foods (list to be supplied), drinking alcohol, and any strenuous exercise the day prior to each visit.
3) We shall also ask you not to consume caffeine from midday the day before each visit and to avoid eating or drinking anything, except water, from after 20.00 h.
4) You will be asked to report to the Metabolic Research Unit in the Diabetes & Nutritional Sciences Division between 08:00 h and 10:00 h, in the fasted state (i.e. without having consumed breakfast and without having consumed any food or drink from after 20.00 h the previous night, apart from water). Make sure you drink some water on the morning of the study to avoid dehydration.
5) At each of the 3 visits, a small flexible tube called a cannula will be inserted in a vein in your arm and a sample of blood will be taken (at baseline 17 ml, or 3½ tsp). Just before this, we will measure the functioning of the blood vessels (DVP) using a sensor placed on your finger, and also your blood pressure. We will make measurements of blood pressure by placing a cuff around your arm which will be inflated. The cuff causes a tingling sensation in your arm, but does not cause pain.
6) You will be given the test meal to consume within 7 min. The test meal on all study days will consist of a fruit drink, consumed immediately before a high carbohydrate meal.
7) Following the test meal we would ask you to stay in the Metabolic Research Unit but you are free to work/read/use your laptop for the remainder of the study day in between measurements.
8) 10 minutes following commencing eating the test meal you will have a second blood sample taken (13 ml/2½ tsp) and further blood samples will be taken at 20 min, 30 min, 45 min, 1 h, 75 min, 90 min, and 2 h after the meal. In total you will have 121 ml/ 24 tsp blood taken on each study day, and up to 380 ml/ 76 tsp blood taken over the course of the study, including the screening visit.

How will this benefit me?

You will have a free health check, including liver function tests, full blood count, blood lipid profile and glucose levels, blood pressure measurements and body composition measurements. Should you wish to find out the results of this study you are welcome to contact Dr Wendy Hall (details below) for a copy of the final report once the study is finished.

Will my participation be kept confidential?

Any information collected about you during this research will be kept strictly confidential. Your GP will not be told that you are taking part in the study, nor will they receive any results from the study, unless you instruct us to provide a letter for you to pass on to them. Subject confidentiality and anonymity will be observed throughout the study by use of subject codes in place of names, and the storage of subject details in a secure place. Only the investigators have access to this data.

What will happen to my study results?

Appendix 5 continued

Your anonymised data will be shared with other researchers. We hope to publish the results of the whole study in a scientific journal. You will not be identified in any publication. We will be happy to discuss the overall results with you when the study is completed, and will let you know how you can get a copy of the published results if you wish.

Who is organising and funding the study?
The study is organised by the Diabetes and Nutritional Sciences Division, Kings College London. In recognition of your time commitment, you will be paid an honorarium of £25 per study day to be paid at the end of the study. Any travel expenses will be refunded for the screening and study visits.

Do I have to take part?
It is up to you to decide whether to take part or not. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You can withdraw from the study at any time by informing one of the researchers and you are not obliged to give a reason. You can also withdraw your data from the study if you wish at any time until the final report is submitted to the funders, which will be 17th December 2015. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

If you decide to take part, please let us know if you have been involved in any other study in the last year.

Thank you for your interest.

For further information, please contact: Monica Castro, Stephanie Stone or Jonathan Mok at glu-frustudy@kcl.ac.uk or by tel. 020 7848 4162, 07526 787103.
Diabetes and Nutritional Sciences Division, King's College London, Franklin Wilkins Building, 150 Stamford Street, London SE1 9NH

If this study has harmed you in any way you can contact King's College London using the details below for further advice and information:

Chief Investigator: Dr Wendy Hall (tel 020 7848 4197, wendy.hall@kcl.ac.uk)

Diabetes and Nutritional Sciences Division, King's College London, Franklin Wilkins Building, 150 Stamford Street, London SE1 9NH

Appendix 6 Telephone questionnaire for participants of randomised clinical trials

Telephone Questionnaire

KING'S College LONDON

Participant details

Date .....

Name ..... M ☐ F ☐

Address .....

.....

Date of Birth .....

Age ..... **EXCLUDE If ≤ 20 or > 61 Years (men), or if <45 or > 61 years (women)**

Women only: Postmenopausal (last period >12 months ago?) Y ☐ N ☐
EXCLUDE if not postmenopausal (women)

Ethnicity .....

Phone Number: Day..... Evening .....

Best time to phone .....

General Practitioner .....

Of (medical practice).....

.....

GP telephone number.....

	YES	NO
Are you a KCL/University of London Employee or student?	☐	☐
Have you taken part in any other research study within the past year, or are you currently taking part in a research study? **EXCLUDE if currently taking part in a trial, donated blood within previous 3 months of screening visit, or if participation in study would result in having donated >1500 mL blood in previous 12 months.**	☐	☐

Details .....

Appendix 6 continued

2

Health

We would now like to ask you some Health questions. If there are any questions you would prefer not to answer please let us know.

Do you smoke? **Exclude if Yes**	YES ☐	NO ☐
Have you recently given up smoking? **Exclude if Yes within the last 6 months**	YES ☐	NO ☐
Do you drink alcohol? If yes, how many units of alcohol would you consume in a typical week? (1 unit = 1 measure of spirits / 1 small glass of wine / 1 half pint of beer) …… **Exclude if current intake of >28 Units/week men or >21 units/week women**	YES ☐	NO ☐
Do you currently take any vitamin, mineral or oil supplements? If **Yes**, please give details. If **Yes** to fish oil supplements, e.g. Cod-liver oil, Omega-3 oils, Eye-Q,	YES ☐	NO ☐
Are you prepared to stop taking certain supplements for the duration of the study? (describe which ones) **Exclude if No**	YES ☐	NO ☐
Do you have any food allergies, sensitivities or intolerances? If **Yes**, please give details.	YES ☐	NO ☐
Are you prepared to stop eating oily fish (mackerel, salmon, etc) for the duration of the study? **N.B.** White and shellfish can still be eaten throughout **Exclude if No**	YES ☐	NO ☐
Are you prepared to stop eating certain fruits and vegetables (berries, grapes, beetroot, apples, etc) for the 24 h before each study day? **N.B.** A list of foods to avoid will be provided **Exclude if No**	YES ☐	NO ☐
Do you know your Body Mass Index? BMI = …… If not, please can you tell us your Weight…… …… & Height…… **Exclude if BMI <20 or >35 kg/m² (to be confirmed at Screening Visit)**		

Appendix 6 continued

3

	YES	NO
Have you had a weight change of more than 7lbs in the past 2 months? **Exclude if Yes**	☐	☐
Do you have phenylketonuria? **Exclude if Yes**	☐	☐

Do you have a history of any of the following conditions?

	YES	NO
(i) Heart attack (myocardial infarction) or stroke	☐	☐
(ii) Cardiovascular problems/angina/thrombosis	☐	☐
(iii) Cancer (exclude basal cell carcinoma) in last 5 yrs	☐	☐
(iv) Diabetes (exclude Type I & Type II)	☐	☐
(v) Stomach or inflammatory bowel disease	☐	☐
(vi) Kidney problems	☐	☐
(vii) Liver disease, adult jaundice or anaemia	☐	☐
(viii) History of drug/alcohol addiction (>60 units/wk)	☐	☐

If Yes to any, exclude from study &/or take clinical advice.

(viii) Chronic medical conditions	☐	☐

Please list any others and give details of recent hospital visits .....

Are you currently taking any medications for the following conditions?

		YES	NO
(i)	Raised Cholesterol or triglycerides e.g. fibrates or statins	☐	☐
(ii)	Raised Blood Pressure e.g. diuretics, β-blockers, Ca-channel, ACE inhibitors, Angiotensin Receptor blockers	☐	☐
(iii)	Immune system, e.g. antihistamines, anti-inflammatory	☐	☐
(iv)	Blood glucose levels e.g. acarbose, metformin, sulfonylureas	☐	☐

If Yes to lipid or glucose-lowering drugs, exclude.
Seek medical advice for other medications.

.....

Please list any other medications (pills, tablets, contraceptives, inhalers etc.) that you are taking, either prescribed by your doctor or purchased over the counter.

.....

	YES	NO
Suitable? **If yes, book Screening Visit**	☐	☐

Date ..... Time .....

ID code G L F ☐ ☐ ☐

Investigator Signature..... Date.....

Appendix 7 Written informed consent form

KING'S College LONDON

CONSENT FORM FOR PARTICIPANTS IN A NUTRITIONAL STUDY

Please complete this form after you have read the Information Sheet and you are satisfied that the research has been fully explained.

Title of Study: The acute effects of blackcurrant and apple extracts on postprandial glycaemia: a randomised controlled trial. The GLU-FRU study

King's College Research Ethics Committee Ref: BDM/14/15-10

Thank you for considering taking part in this research. The person organizing the research must explain the project to you before you agree to take part. If you have any questions arising from the Information Sheet or explanation already given to you, please ask the researcher before you decide whether to participate. You will be given a copy of this Consent Form to keep and refer to at any time.

I confirm that I understand that by ticking/initialling each box I am consenting to this element of the study. I understand that it will be assumed that unticked/initialled boxes mean that I DO NOT consent to that part of the study. I understand that by not giving consent for any one element I may be deemed ineligible for the study.

1) I confirm that I fit into the following criteria:

I am 60 years or younger and I do not smoke	☐
I do not have a history of heart disease, stroke, high blood pressure, diabetes, thrombosis, liver disease, chronic gastrointestinal disorders or a cancer diagnosis (except basal cell carcinoma)	☐
I do not have a history of excess alcohol intake or substance abuse	☐

2) I confirm that I have read and understood the information sheet dated [Version number 20/08/2014] for the above study. I have had the opportunity to consider the information and asked questions which have been answered satisfactorily. ☐

3) I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason. Furthermore, I understand that I will be able to withdraw my data up to 17th December 2015. ☐

4) I consent to the processing of my personal information for the purposes explained to me. I understand that such information will be handled in accordance with the terms of the UK Data Protection Act 1998. ☐

5) I understand that my information may be subject to review by responsible individuals from the College for monitoring and audit purposes ☐

Appendix 7 continued

6) **I agree that the research team may use my data for future research and understand that any such use of identifiable data would be reviewed and approved by a research ethics committee. Please note that in such cases, as with this project, confidentiality and anonymity will be maintained and it will not be possible to identify you from any publication.** ☐

7) **I understand that confidentiality and anonymity will be maintained and it will not be possible to identify me in any publications** ☐

8) **I have informed the researcher of any other research in which I am currently involved or have been involved in during the past 12 months** ☐

Participant's Statement:

I _____

agree that the research project named above has been explained to me to my satisfaction and I agree to take part in the study. I have read both the notes written above and the Information Sheet about the project, and understand what the research study involves.

Signed _____ Date _____

Investigator's Statement:

I _____

confirm that I have carefully explained the nature, demands and any foreseeable risks (where applicable) of the proposed research to the volunteer.

Signed _____Date _____